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THE RELATIONSHIP BETWEEN THE ONSET OF SPEECH AND INTELLIGENCE *

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This paper was suggested because mothers as well as scientific investigators frequently inquire "What is the normal age for speech acquirement and what circumstances determine the early acquisition or retardation of speech?" In order to reply to these questions we asked ourselves "What is the relationship of the onset of speech to intelligence?" Incidentally we were interested in the development of this function in correlation with other static developments of infancy

Our data were procured from the case records on file at the Institute for Juvenile Research in Chicago These records are the carefully prepared case studies of children referred to the institute for examination We are greatly indebted to members of the staff for their painstaking labor in collecting from these records the material for this paper

In consulting a voluminous literature on speech acquisition, it was found that opinions differ as to the age at which this function occurs

Before proceeding in our discussion, we must attempt to decide what constitutes speech function Does it consist in the use of words, syllables or sentences? Stern[1] maintains that the child approaches speech along three paths namely, (1) the active expression of childish babble, (2) unintelligent imitation, and (3) understanding what is said to it As soon as these three actions originally independent of each other, work together, real speech has begun In the statistical data on which this paper is based, the mother was asked at what age the child learned to talk Naturally her answer rested on the observation that speech began when he could use words intelligently and could understand what was spoken to him Stern divides speech development in children into several stages

The Preliminary Stage (first year) Imitation of sound-
a defi first understanding of requests made to the child
progre Period (from 1 year to 1½ years) The child has
that ma few sounds with special meaning which must be
form, h sentences of one word There is as yet no under-
First s grammar or ideas and their significance The
mastered
considered
standing o
words still are nearer to babble, expressed in the form of natural symbols

Second Period (from 1½ years to 2 years) The child realizes the object of speech He has learned that everything has a name and he wishes to master it At this time the vocabulary begins to increase, he begins to inquire as to the names of things He also, at this time, combines several words in the form of short sentences Vocabulary contents are increased first by nouns and then by a large addition of verbs

Third Period (from 2 years to 2½ years) The child learns to express the finer shades of ideas, and modification of words, at about the same time different forms of inflection (conjugation, declension, comparison) begin to develop Series of sentences are formed (exclamations, statements, descriptions questions)

Fourth Period (After 2½ years) The child learns to express thoughts of varying order (principal and subordinate) The child asks questions as to the cause of things

Speech acquirement represents one of the most important functions in human development The mode and period of its acquisition present problems of the greatest interest to the psychologist as well as to the physician Stern remarks that it is to speech alone that man owes the power of all real thought, generalization, judgment, decision, combination, he marvels at the ease and speed of learning to speak

> Compare the effort [he says,] which is expended at school in learning a second language (which is not really mastered even after many years of practice), with, on the other hand the speech of his environment, which seems to flash upon the 2 or 3 year old child without studying any vocabulary or grammar! Month after month he makes the most astounding progress and when he is 4 or 5 years of age, without effort he can make himself understood on every point that comes within his range of interest[1]

The case records of 1,000 white children examined at the Institute for Juvenile Research were read and the following data collected for statistical analysis age of onset of talking, of walking, of bladder control, chronological age, mental age, intelligence quotient, sex, ordinal position among siblings, race nationality of child and of father and mother, and premature birth These thousand cases constitute a somewhat selected group as they are studies of children referred to the clinic for behavior difficulties, as educational problems, for habit training or for advice as to institutional and other placement

Of these records taken from a larger group of cases, 500 were boys and 500 were girls Those records were selected in which the data mentioned were noted, the age of onset of talking being given specifically in terms of months, and in which the children were of the white race When the answer to the question "Age of talking? was indefinite, i e, "about a year," "around eighteen months" "at the usual age," that record was excluded from this study

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* der the Section on Diseases of Children at the Eightieth
he American Medical Association Portland Ore
uly

[1] Stern W Psychology of Early Childhood New York Henry Holt & Co 1924

Not included in the statistical analysis of the 1,000 case records because of their marked divergence from the main group were twenty-one children in whom the onset of talking occurred later than sixty months All of these children had an intelligence quotient below 70 In addition to eleven with gross mental deficiency, there were several in whom delayed speech was attributed to anatomic defects such as cleft palate, deafness or tumor of the tongue

The mental age was acquired by individual psychologic examination on the Stanford-Binet test of general intelligence The intelligence quotient (I Q) is the relation of mental age to chronological age

TABLE 1—*Distribution According to National Origin for Both Sexes*

National Origin	Boys	Girls
Parents American born	151	136
Parents Foreign born	189	141
Jewish	110	175
Unknown	50	48
Total	500	500

The other items were secured from the family and developmental histories of the children Although the reliability of this information is usually dependent on the memory of the relative interviewed, it is fair to assume that the errors in estimation of specific ages neutralize one another in such a large series

The chronological age range for boys extends from 44 months to 23½ years, and for girls from 25 months to 19½ years

The language spoken in the home determined the nationality grouping, but in most instances children coming from homes in which a foreign language was spoken were confronted with a bilingual problem Hence it was decided to group together those whose parents were foreign-born As the Jewish children comprised more than one fourth of the total group and since they constituted a more homogeneous cultural group they were classified separately

In addition, scattered instances of birthplace of parents were given as Greece, Hungary, Austria, Russia, Jugoslavia, Holland, Syria, Roumania and Serbia

The range in intelligence quotient extends from 10 to 159 for both sexes The average intelligence quotient (mean) is 80 for the boys and 82 for the girls, the difference is negligible This rating places the average intelligence for the group at the dull and backward level which is just below the normal This may be the result, in part, of the inclusion of cases referred to the clinic for advice as to placement in institutions for the feebleminded

The criterion for the acquisition of speech was the conscious association of a word with the object In other words the onset of talking occurs at that age at which the child “ utters a sound with full consciousness of its meaning and for the purpose of communication ”[1]

> The time for this beginning of speech occurs shortly after the end of the first year yet individual variations are very marked There are children of 9 months who can already make intelligent use of one word or of several yet there is no cause for anxiety when a child of 18 months still uses nothing but signs It is frequently stated that girls begin to speak somewhat earlier than boys and also that they advance more rapidly in physiological development It is also said that speech development begins considerably later and proceeds more slowly in working class children than in those of cultured circles The child's power of speech, however, after it has once begun, does not have a constant increase from day to day It seems that the general law of rhythm in growth is never so clearly demonstrated as in the progress of speech Periods of stagnation sometimes a source of anxiety to parents, are suddenly replaced by periods of rapid progress, to be followed in turn by another phase of slow progress
>
> Very often this stagnation appears immediately after the *first beginning* of speech The child has, at a comparatively early age gained the intelligent use of one or several words Then months pass without further success, indeed sometimes what he has already acquired is lost again Thus for six months Preyer's son had as his sole vocabulary “atta” (away) and ‘ta” (thank you) while for two and a half months our own son's only word was “da ”[1]

Osnato[2] states that the environmental influence surrounding the child, particularly the amount of care which it receives from parents and relatives, will determine the character of speech development He also thinks that speech understanding may be far in advance of actual speech, for there exists in all children a period when a great deal that is said is understood, but only a small proportion of this can be uttered by the child

The same author believes it a fairly well established fact that there are no sharp lines of demarcation between the various stages of speech development

Villiger believes that the development of speech and intelligence do not have anything to do with each other Indeed, there are many psychologists who hold this view Osnato refutes Villiger's contention and believes that this statement is not logical and is therefore not true He maintains that the development of speech and intelligence go hand in hand and are interdependent to such an extent that they may be, to all intents and purposes, regarded as the same process

Osnato's view in regard to the correlation of speech and intelligence is in agreement with our own observations We believe that speech acquirement occurs in a direct proportion to the degree of mental development We are not unmindful of the fact that variation may occur and that occasionally a bright child may show a tardy development of speech Every physician of experience could enumerate instances in his practice in which a child caused a great deal of anxiety to his parents through delayed speech, though he seemed bright and mentally active in other respects This, of course, does not refer to mentally retarded or deaf children We have encountered instances ourselves in which children learn to talk or make sentences as late as the third or fourth year, who, nevertheless, ultimately prove to be mentally sound and make od progress in their studies We recall the case of whom we treated many years ago who did until she reached her sixth year She sub acquired fluent speech, became a good pupil, and went through high school and colleg siderable credit

TABLE 2—*Birthplace Frequencies of Foreign Born Parents Arranged According to the Sex of Their Offspring*

Country	Boys	Girls
Poland	35	23
Italy	85	20
Czechoslovakia	12	4
Germany	11	11
Scandinavia	8	3
Lithuania	7	5

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[2] *Osnato M* Aphasia and Associated Spee
Paul B Hoeber 1920

We have a letter from a mother who gives the story of speech acquirement of her 13 year old child. At a year and a half he could say "mamma" and "da-da" and a few other words. He walked at 23 months. His family called him "dummy" at 2 years of age because his vocabulary was limited to only a few words. At 3 he started to make simple one or two word sentences and by the fourth year he had not made any appreciable progress. After a severe illness with influenzal pneumonia in his fourth year, he began to improve and display average intelligence. By the sixth year he read very well, added, subtracted, multiplied and divided any number up to ten. He made his first two grades of school in one school year but he did not make very favorable progress in the third grade; from the fourth to the eighth grade he was either first or second in his class. He made averages of from 92 to 100 and graduated from grammar school at 12 years and 2 months. He was the youngest child, had the highest average and was president of his class. Today, at 13 years and 3 months, he is on the honor roll in high school, is a fair pianist and excels in the study of foreign languages.

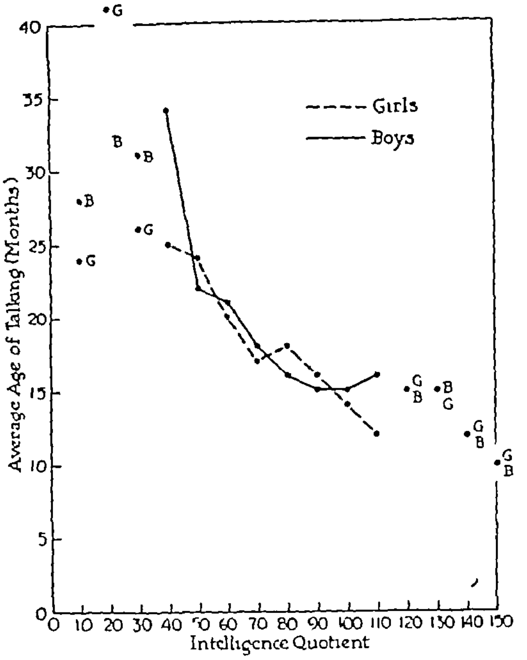

Chart 1.—Relationship between age of talking and intelligence quotient.

There is no doubt that there are exceptional cases children who, later in childhood, show considerable intelligence but have learned late to express themselves by language. Such cases present numerous factors to be considered. Nutritional disturbances, somatic diseases and, as has already been pointed out, unfavorable environment may in some instances influence delayed speech production.

Treitel[3] says that the urge to speak is dependent on a definite stage of mental development. Should this progress slowly, speech will appear slowly. He thinks that mental progress is sometimes slow in rachitic children. Kurt Huldschinsky[4] believes that the ability to talk is inhibited in rachitic children. He maintains that the delayed speech function goes hand in hand with the delayed static functions. He expresses the view that rachitis has some obscure influence on those mental processes which manifest themselves in a state of negativism; this occurs most markedly in the second year of life. These children, for apparently no good reason, resist every approach, object to the most trivial examination, refuse to offer the hand, and often will not utter a sound or a word. For the same reason they might refuse to partake of food. We are not willing to accept these peculiarities in behavior as rachitic manifestations for perhaps they are due to more complicated psychic disturbances which cannot be satisfactorily explained here.

It may be assumed that occasionally there is delayed development of the speech center and association tracts though of course, this would be difficult to prove. It has also been stated that in some cases delayed speech is due to physiologic laziness or perhaps to absence of the "sprach-lust." Others have said that some children are normally bright though they fail to concentrate or are too much absorbed by play and are bored by attempts to talk. These are merely assumptions which are not founded on proved facts.

The range in the age of onset of talking extends from 6 to 60 months for both boys and girls. The average (mean) age of talking for the boys is 19 months and for the girls is 18 months. The earlier age at which girls learn to talk bears out the observations of Mead,[5] Smith[6] Stern[1] and Nadoleczny.[7]

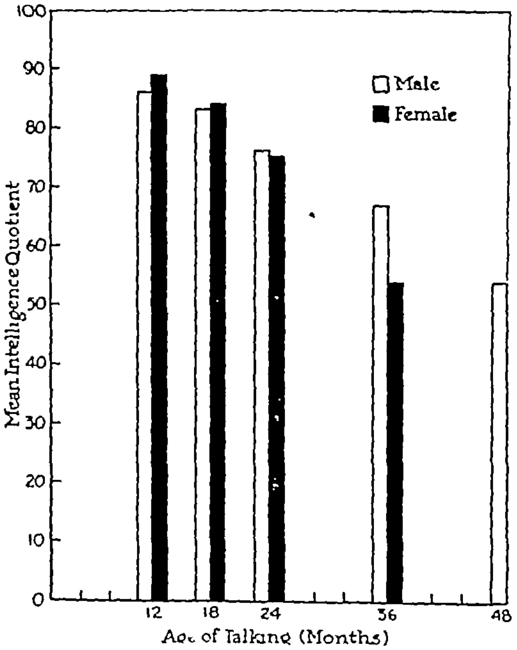

Chart 2.—Relationship between mean intelligence quotient and age of talking.

When the relation between the age of speech onset and the intelligence quotient is determined, coefficients of correlation (Pearson product moment formula) result in —.41 ± 0.03 for the boys and —.39 ± 0.03 for the girls. In view of the low probable error, the correlations are quite reliable and may be interpreted to mean that there is a definite relationship between these two factors

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[3] Treitel Leopold Über aphasia im Kindesalter Klin Vortr a. d Geb d Otol u Pharyngo-Rhinol 64 1893

[4] Huldschinsky Kurt Dementia rachitica Berlin S Karger 1926

[5] Mead C D The Age of Walking and Talking in Relation to General Intelligence Pedagog Seminary & J Genet. Psychol 20 460-484 1913

[6] Smith Madorah E. An Investigation of the Development of the Sentence and the Extent of Vocabulary in Young Children University of Iowa Studies in Child Welfare 3 1 92 number 5 1926

[7] Nadoleczny M Die Sprach und Stimmstorungen im Kindesalter in Pfaundler and Schlossmann Handbuch der Kinderkrankheiten Leipzig F C W Vogel 5 1926

Chart 1 expresses this relationship graphically and shows that the higher the intelligence quotient (that is, the more intelligent the child) the earlier it learns to talk The drop in the curve is steep, as the intelligence quotient increases, the age in months at which a child first talks decreases The few scattered cases at each end of the curve bear out the general trend

TABLE 3—*Analysis of Data According to National Composition*

		Non-Jewish		
		American Born	Foreign Born	Jewish
Coefficient of correlation speech onset	♂	—3 ± 0 05	—40 ± 0 04	—47 ± 0 05
intelligence quotient	♀	—3 ± 0 05	—38 ± 0 05	—43 ± 0 04
Average age of speech	♂	19 months	20 months	18 months
onset	♀	19 months	20 months	16 months*
Range in age of speech	♂	6-60 months	6-60 months	7-48 months
onset	♀	6-60 months	6-60 months	6-60 months
Average intelligence	♂	82	73	89
quotient	♀	80	70	90
Number of cases	♂	161	189	110
	♀	136	141	175

* In this column ♂ stands for male ♀ female

Chart 2 shows the more significant ages at which the onset of speech occurs It includes those children who learned to talk at 12, 18, 24, 36 and 48 months It presents the average (mean) intelligence quotient for each one of these age groups As in the previous chart, it may be seen that those children who learn to talk earlier are brighter

In view of the national composition of the group, it was decided to analyze the data on that basis The results are presented in table 3

The coefficients of correlation between age of speech onset and the intelligence quotient are uniformly high for all three groups and indicate a close relationship between these factors

The average age of speech onset is lowest in the Jewish group and highest in the group with both parents foreign born That is, the former tend to talk at an earlier age while the latter learn to talk later than those of the third group—the children whose parents are born in the United States The range in age of speech onset is about the same for all groups

The average intelligence quotient is highest for the Jewish group and lowest for that whose parents are foreign born Many of the Jewish children referred for study come through one of the scholarship agencies which is primarily interested in selecting the brighter children for the advantages of a higher education The lower average intelligence quotients of the children of the foreign-born parents are partially the result of the language factor in the Stanford-Binet test Vocabulary and language usage play an important part in this test and children who come from a home in which a foreign language is spoken are under a handicap

Onset of walking marks another important stage of development of the static functions Since onset of walking and talking are frequently associated, a brief study of the former is here included Analysis of the thousand cases shows that the average age of onset of walking for both sexes is 16 months The range is 7 to 51 months for boys and 7 to 60 months for girls When the age of onset of walking is correlated with the intelligence quotient, the correlation (the Pearson product moment formula) for boys is —36 ± 0 03 and for girls is —37 ± 0 03 This shows that there is a definite relationship between the age of onset of walking and general intelligence The average age of onset of walking is several months earlier than that of talking This corroborates the observations of Mead

Chart 3 graphically presents the significant relationship between age of onset of walking and general intelligence The more intelligent children learn to walk earlier The slope of the curve has the same general trend as that showing the relationship between age of speech onset and intelligence quotient The curve consistently falls several months below that for the onset of talking, this means that children of the same general level of intelligence learn to walk earlier than they learn to talk

As certain theories have been advanced as to the influence of older siblings on the age at which children learn to talk, this point also was investigated Many believe that if a child has older brothers and sisters he will learn to talk earlier The boys and girls of this group range from an only child to one of thirteen siblings The average position in the family for the thousand cases is 2 8 In this study there was found absolutely no relationship between the ordinal position of a child among his siblings and the acquisition of speech The coefficient of correlation is 0 03 ± 0 03 for boys and 0 00 ± 0 03 for girls

Out of 158 children who had enuresis at the age of 3 years or above the average age of learning to talk is a little more than 21 months Of the seventy children who, according to the case record, established dry habits *prior to the third birthday, the average age of learning* to talk is only 17 months This is an average difference of more than four months and is statistically significant in that this difference is about 4 7 times the size of its probable error According to the tables of the probability integral, the chances that a difference of this magnitude could have arisen purely from random sampling of material which is actually uncorrelated is only once in about 625 trials and is obviously a small probability

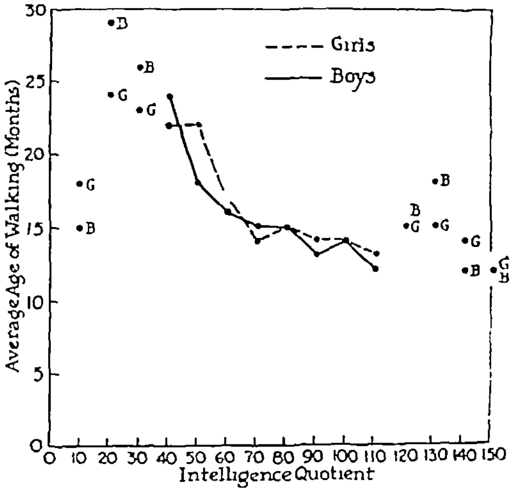

Chart 3—Relationship between age of walking and intelligence quotient

While the distinction between children suffering from enuresis and those not troubled with this condition is made here on the basis of three years, we found that if we had taken five years as the dividing line the difference would remain about the same

We might add as a point of interest that in studying a small group of prematurely born children we found that the average age of speech onset was not appreciably different from that of the main group (the formula for comparison of two means by "Student") We found here, too, that the age of speech acquisition varies in indirect ratio to general intelligence

SUMMARY AND CONCLUSIONS

1 The case records of 1,000 white children who were referred to the Institute for Juvenile Research for examination were studied with a view to determining the relationship between the onset of speech and general intelligence

2 The group studied was composed of 500 boys ranging in age from 3 years 8 months to 23 years 7 months at the time of examination, and of 500 girls between 2 years 1 month and 19 years 5 months

3 More than one fourth of these children had parents who were born in America, more than one fourth were Jewish, and about one third had foreign-born parents Fifteen European countries were represented among the birth places listed

4 On the Stanford-Binet test for general intelligence the intelligence quotients ranged from 10 to 159 The average intelligence quotient for boys was 80 and for girls was 82 The average intelligence rating of the group was slightly below normal

5 The average age of speech onset was 19 months for boys and 18 months for girls When age of speech onset was correlated with the intelligence quotient, the coefficients were —41 ± 0 03 for boys and — 39 ± 0 03 for girls There is a definite relationship between these two factors

6 The average age of onset of walking was 16 months for both sexes The coefficient of correlation between age of onset of walking and intelligence quotient was — 36 ± 0 03 for boys and — 37 ± 0 03 for girls The relationship between these two factors was significant

7 The ordinal position of the children among siblings did not have any relationship with the age of onset of speech

8 Children who are enuretic at the age of 3 years or above learn to talk later than those who have established dry habits prior to that time The average difference in the age of speech onset is more than four months

9 The observations in a similar study made on thirteen children prematurely born were not appreciably different The age of speech onset varies in indirect ratio to general intelligence.

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ABSTRACT OF DISCUSSION

Dr H L Moon, Seattle The authors have taken us over into the realm of child psychology along with our medical work in the examination of these thousand children. The earliest vocalization in children is inarticulate speech with more or less random movements and gestures, in what they called the babble with unintelligent imitation Next is a recognition of words before utterance then speech progresses from babble and cries to words at the age of 1½ years, and from words to sentences by the third year Words are spoken in the fifth year without baby talk A language is acquired by imitations The child has articulate language from about the second to the seventh year and that is the age of perception and retentive memory in the infant. Earlier the child distinguishes between sound and speech There is a certain vibration pattern that is, the child recognizes the parent s voice, and then the voices of members of the family As to speech itself, it is said that speech coordinates the activity of men The fundamental idea of speech is to gain some desire or to satisfy some emotional requirement, and as a source of pleasure to the speaker In the evolution of speech, we can trace it down from our arboreal ancestors who used certain guttural sounds or certain speech in the tribe or group in order to direct the chase to get meat for the tribe, or as a warning to get out of the way in case of danger In the infant, certain sounds seem to give a sense of power as far as speech is concerned The repetition of these sounds brings certain desired results, and further experimentation with sounds leads to speech through imitation This type of child develops speech rapidly in comparison to the shy nervous child, or the neglected child who is put off to one side, and possibly does not have occasion to hear a language as it should be spoken or does not pick up or have a chance or the ability to pick up, as does the child who has good parents to imitate and can handle speech as he collects it very rapidly Helzer and Rundorff show a difference between the professional class and the worker s child, as the authors have brought out. In their instinctive utterance they are about the same, that is, both groups respond at the same age to hunger and pain The professional class seems to be ahead of the working class, in vocabulary, anywhere from nine to twelve months, and in sentence usage, *in inflection or in* parts of speech up to fourteen months Alphabetical sounds are not the only expression. Each infant has a different type of cry The crooning of mother and child is not true speech but the sounds seem to be a source of pleasure and gratification From the crooning the child goes on to what might be called a form of autopoetic type of speech Instead of naming animals the child will say "baa-baa' for sheep, or 'moo moo" for cow After that the child approaches what I should call a "whazzat' age In other words, he wants to know the names His speech is not good as yet, but everything he points to is "whazzat"

Dr I Leon Meyers, Los Angeles It is generally agreed that the various phases in the evolution of a function are duplicated in the dissolution of the function In my work in clinical neurology I deal more with the disintegration of speech as this manifests itself in aphasia than with the acquisition of speech in childhood. And I should state without hesitation that my experience fully substantiates the conclusions of Drs Abt and Adler in their study of the latter condition Aphasia, as is well known, has been for a long time divided into the motor type and the sensory type, and it was thought that the motor type frequently coexists with normal mentality This was denied by Marie, who maintains that aphasia invariably implies a loss or an impairment of mentality This view is undoubtedly correct, and we are warranted in concluding that a delay in the acquisition of speech in childhood means a delay in the acquisition of intelligence. There is, it is true, a form of speech loss known as anarthria in which the patient cannot articulate, although his mentality is normal The condition is brought about by an impairment of function of the pyramidal tracts In the case of a child such a condition may be the result of a delay in the maturation of these tracts The child affected by such a pathologic process may even be able to use his tongue and palate for such coarse movements as sticking out the tongue and swallowing He is unable, however, to use these muscles for the finer movements of articulation This is undoubtedly the condition in the few isolated instances in which the child is unable to speak although possessed of fair intelligence but the fact that the pyramidal tracts are delayed in development generally indicates that there is something wrong with the child s mentality

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Mental Test Movement Wanes—The mental test movement which made its bid for the solution of all mental problems by a cheap and simple method short-circuiting the laborious laboratory method has suffered notable changes The points which were clear to all of the laboratory men fifteen years ago are now painfully apparent to those serious psychologists who were for the time dazzled and the others are hastily preparing alibis and seeking cover The astonishing doctrine that intelligence tests measure mental capacities directly once broadcasted from centers of authority is now heard only in the echoes from the more remote places and mental testers are beginning to understand that only acquisitions are measured—Dunlap, Knight The Outlook for Psychology *Science* Feb 22, 1929

CANCER OF THE LOWER END OF THE ESOPHAGUS AND CARDIAC END OF THE STOMACH *

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In a former paper I called attention to the fact that cancer of the esophagus is a subject to which the medical profession cannot point with pride, as the diagnosis is usually made very late in the history of the case and the diagnosis all too frequently is the profession's only contribution in its clinical course

Cancer of the upper esophagus does not lend itself to early diagnosis by means of the x-rays for the obvious reason that it is difficult to fill the lumen of the upper esophagus with the contrast meal Fortunately the lower and middle thirds of the esophagus, where distention with the contrast meal is more easily produced, are more favorable sites for accurate examinations The direct demonstration of the cancer without a barium filling is of little value and will not be considered It is best to study by a contrast meal any encroachments by new growths on the lumen of the lower esophagus If the usual fluid suspension of the barium mixture is employed, it will be found that the passage of this thin mixture through the esophagus may take place so rapidly that doubt may remain as to whether even moderate sized new growths may be detected A thick mixture of mucilage of acacia with barium will follow down through the esophagus much more slowly and may thus bring into relief a moderate sized cancer However, the difficulties of the oblique positions and their confusing mediastinal shadows all detract from the desired accuracy

The method devised by Dr William H Stewart of using a sausage casing as a container for the barium is ingenious and satisfactory, though it frequently produces nausea and vomiting The right oblique prone position of Manges permits of a better distention of the esophagus with a longer delay before the opaque mixture passes on into the stomach, giving the roentgenologist more time to study the contour of the esophagus and any encroachment on its lumen

These methods have proved satisfactory for the detection and the demonstration of advanced lesions In 1925, Dr Chevalier Jackson[1] accentuated the importance of a diagnosis earlier than is usually made He graphically emphasized the uselessness of paying attention to symptoms which are characteristic only of the far advanced neoplasm Pronounced difficulty in swallowing with marked loss of weight are symptoms which should not be waited for before one resorts to roentgen examination and esophagoscopy We would not get far in the treatment of cancer of the lip or in the treatment of cancer of the breast if we waited for ulceration and loss of weight If physicians are to accomplish anything in the treatment of cancer of the esophagus, the diagnosis must be made before pronounced constitutional symptoms have developed

For the detection of early lesions of the middle and lower esophagus I have found the use of the hard gelatin capsule filled with barium, the elastic gelatin capsule and the barium gelatin bougies of value By giving the patient gelatin capsules, beginning with the smaller sizes and increasing the diameter until obstruction is met, an accurate idea can be obtained of the site of the obstruction The hard gelatin capsule does not conform to the irregularity of the esophagus and is therefore of lessened value It presents, further, the grave objection that if it becomes lodged in the esophagus it may remain for a number of hours with considerable discomfort to the patient The elastic capsule of softer gelatin filled with barium paste presents advantages over the hard capsule but is difficult to obtain Therefore I call attention to a method which I have employed for several years with considerable satisfaction The principle of the method is the use of soft gelatin bougies containing barium These bougies are easily prepared and the technic of their preparation is as follows

Ordinary cooking gelatin is allowed to soak in cold water until it swells fully, after which it is rendered fluid by heating over a water bath When the gelatin has become thoroughly liquefied, barium paste made by thoroughly mixing barium with a small amount of water is added and thoroughly stirred The mixture of the liquefied gelatin and barium is then poured into a cup and allowed to cool After the barium gelatin is thoroughly jellied, it is removed from the cup and with brass cork borers of varying diameter plugs are cut out of the gelatin These plugs usually are from 3 to 4 cm long and from 8 to 16 mm in diameter

The patient is then studied with the fluoroscope as he swallows with the aid of water these gelatin bougies If one becomes lodged, the heat of the body will cause it to soften after two to three minutes and the barium gelatin will pass down into the stomach If there is a tendency for the barium gelatin plug to stick, it can be at once softened and dislodged with a drink of warm water These barium gelatin bougies are easily swallowed by the patient and even slight projections into the lumen of the esophagus can be easily detected It is of equal importance that a negative diagnosis can be made if the larger sizes are easily swallowed and pass uninterruptedly through the esophagus Previous to their employment I always felt diffident in making a negative diagnosis for cancer

I submit this method as a contribution to the early diagnosis of cancer of the lower esophagus If the gelatin plug sticks at any point, the size and contour of the esophagus above the point of constriction can be studied for a moment or so by giving the patient a thin mixture of barium This thin mixture will fill the esophagus above the plug and even slight dilatation can be studied in this way As a further refinement in their use, a piece of string can be threaded through the plug To this string, which is prevented from pulling out by a knot in the string with a small washer of thin wax paper, a small silk thread is attached When the patient swallows this bougie, the plug may be observed in its passage down and may by tension on the thread be raised up to points where further study is desired This procedure is a complement of esophagoscopy I feel that the necessity for esophagoscopy will not arise if the patient can comfortably swallow the larger sized bougies, say up to 14 mm in diameter If, however, the patient shows a tendency to even slight obstruction made evident by fluoroscopic observation of the gelatin bougies, esophagoscopy can then be a supplementary method of diagnosis I feel quite convinced that this method of the gelatin bougie presents advantages in assisting in the establishment or refutation of the diagnosis of an early cancer of the esophagus

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* From the University of Michigan Medical School

* Read before the joint meeting of the Section on Gastro-Enterology and Proctology and the Section on Radiology at the Eightieth Annual Session of the American Medical Association, Portland Ore. July 11 1929

[1] Jackson Chevalier Carcinoma and Sarcoma of the Esophagus A Plea for Early Diagnosis Am J M Sc. **169** 625 (May) 1925

In the fluoroscopic examination of the esophagus, the right oblique or the left oblique position will visualize the lower esophagus without the obtrusive shadows of the spine and heart The left oblique position, with the patient examined during full inspiration gives a clearer view of the lower esophagus than any of the other projections Another method which I have found to be of advantage is the examination of the patient lying on his left side with the tube placed posterior and the fluoroscopic screen against the anterior lower chest wall For this a narrow table is employed and placed against the front of the vertical fluoroscope The patient lies on his left side and is allowed to swallow the opaque meal through a feeding spoon during fluoroscopic observation In this position the outline of the lower portion of the esophagus can be thoroughly studied and the relation of the esophagus to the stomach well brought out

The fluoroscopic appearance of cancer of the lower esophagus shows usually an irregularity of the lumen This irregularity may be covered up, however, by an attendant spasm producing a dilatation which may partially hide this defect The irregularity may be evident by an encroachment on the lumen or by a deviation from the usual course In examining patients during suspended respiration in full inspiration, one should remember that there is always a physiologic break in the course of the passage of the barium This appearance is physiologic and is caused by the pressure of the diaphragam in most patients and should not be confounded with evidence of a pathologic condition The immediate test, of course, is to allow the patient to breathe and with the relaxation of the diaphragm as expiration begins, the course of the barium is at once resumed

Roentgen signs of esophageal cancer at the cardia are

1 Delay in the bismuth meal with dilatation above (late)
2 Irregularity of the lower esophageal outline
3 Visible peristalsis in order to overcome the obstruction
4 Usually a trickle of barium past the point of narrowing (late)
5 Failure of large barium bougies to pass

The roentgen examination of the stomach bears a certain relationship to the roentgen examination of the esophagus in that neoplasms of the upper third of the stomach are shown with greater difficulty than neoplasms in the lower third This follows as a consequence of the greater difficulty of fully filling the upper third of the stomach with the contrast meal Of especial value in this connection is the article which Dr Carman presented in 1923 before the American Gastro-Enterological Association entitled "Technical Aids in Roentgenologic Demonstrations of Lesions High in the Stomach and on the Posterior Wall"

The radiologist acknowledges a higher percentage of error in lesions of the upper third of the stomach than in the diagnosis of pathologic conditions in the middle and lower third in routine examinations Therefore, the difficulties of visualization of filling defects in the upper third of the stomach must be met by a special technic First of all, one must carefully observe fluoroscopically the behavior of the bismuth mixture as it spurts from the esophagus into the stomach Its course should be carefully observed and any deviations from the usual channel should be noted This fluoroscopic examination should if possible, be supplemented by serial films In routine examinations, observations in the erect and prone positions should always be supplemented by examination in the supine position

Further, all gastric examinations in patients of the cancerous age should include an examination of the patient in the Trendelenburg position so as to insure as complete a filling as possible of the upper pole of the stomach Patients showing increased gastric motility should always have an exhaustive survey made of the upper third of the stomach This survey should be both fluoroscopic and with serial films There is always a possibility of missing lesions in the upper pole of the stomach if one relies on serial examinations made in the prone position I have found the use of the narrow table placed in front of the vertical fluoroscope to be of great value in completely outlining the greater curvature in its upper portion With the patient lying on his left side and given the opaque meal while in this position, defects on the greater curvature can be more satisfactorily demonstrated I would call attention to the frequency of a carcinoma involving the upper pole of the stomach and extending by direct continuity into the esophagus The clinical history in these cases is oftentimes fairly characteristic, the patient giving a history which simulates that of gastric ulcer succeeded by a period in which dysphagia is added to the symptoms

In a recent study of thirty-two cases of malignant lesions of the lower esophagus and upper third of the stomach seen at the Michigan University Hospital, compiled by my assistant, Dr C L Gillies, it was found that in sixteen of these cases the growth was primary in the upper pole of the stomach and then extended directly into the esophagus One case was interesting in that the gelatin bougie detected a slight narrowing of the lumen of the lower esophagus while the examination of the upper third of the stomach showed an extensive infiltration on the posterior wall In another case in which we found decisive evidence of cancer of the lower esophagus, the roentgen indications of lesions in the upper third of the stomach were inconclusive Afterward a gastrostomy and manual exploration revealed an extensive gastric carcinoma Barium was introduced into the stomach through the gastrostomy tube, and even with the patient in the Trendelenburg position an outline of the lesion was difficult to obtain and did not correlate with the exploratory observations

Roentgen signs of gastric cancer at the cardia are

1 Irregularity in the spurting of the barium from the esophagus into the stomach
2 Irregularity in the outline of the cardia in the Trendelenburg or left lateral position.
3 Constancy of deformity in serial films
4 Marked gastric hypermotility

I feel that the cardiac portion of the stomach is a region which presents difficulties in the early detection of cancer In every case presented for examination the possibility of an early cancerous lesion should be considered, and therefore all the refinements of the radiologic art should be employed

SUMMARY

1 The usual methods of roentgen examination of the esophagus are often insufficient to detect small new growths

2 The gelatin barium bougie is a delicate test for encroachment on the lumen of the esophagus and may furnish reliable data for a negative diagnosis

3 The barium gelatin bougie is of importance in determining the desirability of an esophagoscopy

4 In the endeavor to detect early malignant lesions of the lower esophagus and of the cardiac portion of

the stomach, all the special technics at the disposal of the radiologist should be employed.

5. The examination of the patient in the recumbent left lateral position is often advantageous.

6. We should be unceasing in our endeavor to perfect our technic in the hope of making diagnosis much earlier than is now done.

ABSTRACT OF DISCUSSION

Dr. B. H. Orndoff, Chicago: It has been almost a hobby with me to try to gain technical points in the diagnosis of abdominal conditions by rotating patients in different positions and observing the organs filled with opaque substances with the source of light passing through the body at different angles. We adopted at first the method described by Dr. Hickey, using a small narrow table in front of an upright screen. We saw the advantage and the necessity almost of adding a feature to the regular hospital cart or table, an arrangement that would permit elevation of the hips above the shoulders to a marked degree, and vice versa, a partial elevation of the shoulders above the hips. This increased our interest until about 1919, when pneumoperitoneum came along; then we felt keenly a demand for a special equipment that would give us these positions accurately. A table has been developed by the Victor Electric Corporation in which the tube and the screen are supported on two circular wheels which surround the table making it possible to move the source of light from one plane to another and hold the patient in the same position. On that same table we can elevate the hips above the shoulders or the shoulders above the hips and when the proper position is found at which an organ remains filled or partly filled, we can vary the source of light in the different directions so that we can bring out a defect that might be obscure otherwise. Another point which the author failed to stress is the advantage to be gained by the air bubble in the stomach. In some of these cases with pathologic changes in the lower end of the esophagus and upper cardia a very small air bubble will be found in the stomach. We have found it necessary to try to reduce that in some cases, and in some cases have actually caused a pressure above the lesion by inserting a gastric tube and pumping air into it until we got enough air in the stomach to show plainly. One can throw the gas bubble into any portion of the stomach desired simply by turning the patient. Sometimes there will be hernia pouches or ulcers that have suppurated, and by having the patient in the direct posture there is a filling of the stomach in the usual way, while with the face upward or downward with the usual equipment one would miss it altogether. This, of course, demands painstaking technic. It is time-consuming and it becomes more or less irksome from that standpoint. But I believe that in the difficult cases, in which the radiologic observations do not explain the clinical signs, we are obliged to keep on with the search until we can eliminate every line of investigation that comes to us.

Dr. Frank Smithies, Chicago: While the other regions of the stomach and the duodenum are pretty well taken care of by the roentgenologists, it has been my experience that our errors and their errors have occurred in these esophageal or high gastric lesions. This has certain practical points. Dr. Hickey mentioned that one third of the instances which were considered to be cases of carcinoma of the esophagus were primarily carcinoma of the stomach. We have seen a number of instances in which, at a great sacrifice of time and money, the patients have been subjected to radium treatment of the lower esophagus for the curing of esophageal carcinoma, when the lesion was really a hopeless gastric cancer. The patients did not receive any benefit, and in two instances, at least, through such treatment, died of hemorrhage or perforation of the high lying gastric carcinoma. The plates that come to us which are negative when a lesion exists are usually of those patients whose stomachs have not been completely filled by barium. All of us cannot avail ourselves of the technical assistance by special apparatus that Dr. Hickey and Dr. Orndoff mentioned, but at least we can all insist that we give no diagnostic opinion regarding the stomach unless the stomach is completely filled. If this is done fewer high-lying lesions will be missed. Another point is the physical evidence of a tumor high in the stomach. I mentioned a number of years ago that in such lesions, if the patient was placed flat on his back, with his hips elevated, there would be dulness in Traube's space on expiration. When the patient inspires deeply, Traube's space becomes tympanitic to percussion.

Dr. George B. Eusterman, Rochester, Minn.: When one has had the valuable cooperation of an expert roentgenoscopist there is a great deal of security in the often necessary rapid turn over of patients, in the satisfactory elimination of intragastric lesions, especially with repeated negative fluoroscopic examinations. Nevertheless, when the clinical symptoms and signs are suggestive of an intra-abdominal malignant growth, especially in the stomach the possibility of being unable to visualize lesions in the upper third of the stomach, particularly on the posterior aspects of that organ, is considerable. The history of recent dyspepsia in an elderly adult, associated with loss of weight, anemia and the presence of fresh and altered blood in the gastric contents or in the stool on a meat-free regimen justifies repeated roentgenoscopic reexamination under such circumstances. It is a curious fact that such gastric lesions difficult of visualization are usually inoperable anyway, but in spite of that one should have the satisfaction of making a definite diagnosis in the interest of the patient, if for no other reason. We have had a number of experiences in which withdrawal of the stomach tube brought with it malignant tissue and yet a negative fluoroscopic report was returned on the initial examination. It is necessary to do an exploratory operation in some of these cases to confirm the clinical diagnosis when the laboratory procedure does not help solve the problem. Experiences in this group of cases show us that there is no royal road to diagnosis.

Dr. E. D. McCarty, Tacoma, Wash.: Dr. Hickey did not mention that in making a gastro-intestinal examination one should always place the patient in the horizontal position, then have him take a deep breath. If the dome of the diaphragm outlined by the barium-filled fundus of the stomach, does not change its shape one is suspicious of the presence of malignancy. Another point is that in the upright position I have noticed the fundus of the stomach stooling over the media, suggesting an incisura. In those cases I am always suspicious of a lesion in that region, and by turning the patient I am often enabled to bring this out, but by taking a film with the patient perfectly flat on his back and his feet slightly elevated, with a heavy exposure, when there is not too much barium in the mixture, one will bring out a mottled condition up in the fundus, or some definite area of dense barium, while the malignant growth will show along the upper margin of the fundus, the rugae showing up through the barium except where the barium is blotted out by the deep exposure.

Dr. P. M. Hickey, Ann Arbor, Mich.: I would like to emphasize the point brought out about repeated examinations. The physician, when he examines, says, 'I will come in tomorrow and see you again.' He goes back with his stethoscope the second day. I think the same courtesy should be given the roentgenologist to repeat the examination either to confirm the negative diagnosis or, perhaps, to add to it. The table which Dr. Orndoff devised some years ago is a useful piece of apparatus, and I am sorry there are not more of them in general use because it permits placing the patient in advantageous positions. The table which I demonstrated was made by the hospital carpenter. The central section is 36 inches long. There is a piece 18 inches long at each end which will drop down out of the way when one is not using the table, and which can be brought up to a horizontal position and held by a little support on each end. In that way one has a table 6 feet long which when not in use, can be folded down and placed to one side. We have found it useful in making examinations of the stomach and the esophagus and also in the detection of changing fluid levels in examining the thorax. I think that the use of these simple aids in the laboratory often helps in some of these rather difficult diagnoses. I will say again that I think the roentgenologists have been missing too often the lesions in the upper third of the stomach and therefore I think that if the physician does not give his clinical data, the roentgenologist should have a conference with the patient so that he may proceed with his work in a more orderly and understanding fashion.

TREATMENT AFTER PROSTATECTOMY *

L P PLAYER, M D
SAN FRANCISCO

The preoperative preparation of patients about to undergo prostatectomy is understood by urologists in general, and the medical literature is liberal in its exploitation One may conservatively state that the present low mortality rate is largely dependent on such preparation One finds, however, instances in which, after the incision has healed and voiding of urine has been established, the general condition of the patient is below his preoperative standard If infection did not exist previous to the operation, the bladder becomes infected following operation In many cases because better drainage has been established by surgery, the urine may become sterile under simple urinary antiseptics, bladder lavages, or by treatment directed to infected foci elsewhere Again, the bladder infection may persist and defer convalescence

Table 1—*Data on First Ten Cases*

Case	Residual Urine		Laboratory Observations							Permanent Phenolsulphonphthalein Maximum				Average Temperature		General Condition	Epididymitis	Rectal Observations Palpable Vesicles Perivesiculitis
				Postoperative						Preoperative Average		Postoperative Average						
				Bladder		Seminal Vesicles		Kidneys										
	Preoperative	Postoperative	Preoperative Bladder	Infection	Pus	Infection	Pus	Infection	Pus	1st Hour	2d Hour	1st Hour	2d Hour	A M	P M			
End of First Quarter																		
A	70 cc	0	Streptococcus viridans and diphtheroid	++	+	+	++	0	0	85%	20%	30%	25%	97.8	100	Fair	0	Painful and indurated to palpation in all
D	160 cc	0	0	++	+	+	++	0	0	85%	15%	40%	15%					
F	55 cc	0	B coli and Streptococcus viridans	++	+	+	++	0	0	35%	25%	35%	25%					
G	560 cc	0	Streptococcus non hemolytic	++	+	+	++	0	0	30%	30%	40%	20%					
K	90 cc	20 cc	Streptococcus viridans	++	+	+	++	0	0	30%	20%	35%	10%					
M	55 cc	20 cc	Staphylococcus and streptococcus	++	+	+	+++	0	0	50%	25%	40%	20%					
O	700 cc	40 cc	B coli	++	+	+	+++	0	0	85%	20%	35%	25%					
End of Second Quarter																		
A		0		++	+	+	++	0	0					More normal		Improved	0	Improved
D		0		+	+	+	++	0	0									
F		0		+	+	+	++	0	0									
G		0		+	+	+	++	0	0									
K		20 cc		+	+	+	++	0	0									
M		20 cc		+	+	+	++	0	0									
O		20 cc		+	+	+	++	0	0									
End of Third Quarter																		
A		0		0	+	+	+	0	0					Slight variation		Greatly improved	0	Improved
D		0		+	+	+	+	0	0									
F		0		+	+	+	+	0	0									
G		0		+	+	+	+	0	0									
K		0		0	+	+	+	0	0									
M		20 cc.		+	+	+	+	0	0									
O		30 cc		+	+	+	++	0	0									
End of Fourth Quarter			O still under observation							A D G E M K discharged at end of eighth quarter								
End of First Quarter																		
B	610 cc	10 cc	B coli and Streptococcus nonhemolytic	++		++++		0		30%	20%	30%	20%	98.6		Good	0	
F	1 450 cc	60 cc	B coli and diphtheroid	++				0		15%	25%	35%	10%	97.6	99	Poor	0	
J	870 cc.	10 cc	Streptococcus non hemolytic and Staphylococcus albus	++				0		35%	10%	15%	20%	98	99	Good	0	
End of Second Quarter																		
B		10 cc				Improving						55%		Normal		Good	0	
F		60 cc				No improvement						45%		98	100	Poor	0	
J		10 cc				Improving						50%		Normal		Good	0	
End of Third Quarter																		
B		10 cc				Improving						55%		Normal		Good	0	
F		60 cc				No improvement						45%		98	100	Poor	0	
J		10 cc				Improving						50%		Normal		Good	0	
End of Fourth Quarter																		
B						Improving						55%		Normal		Good	0	
F		60 cc				No improvement						45%		98	100	Poor	0	
J						Improving						50%		Normal		Good	0	
End of Fifth Quarter																		
B		0				Improving								Normal		Good	0	
F						No improvement						+50%		98	100	Poor	0	
J		0				Improving								Normal		Good	0	
End of Sixth Quarter																		
B						Improving								Normal		Good	0	
F		60 cc				Improving								98	100	Poor	0	
J						Improving								Normal		Good	0	
End of Seventh Quarter																		
B						Practically normal								Normal		Excellent	0	
F		40 cc				Infection ++ pus +								98	100	Improved	0	
J						Practically normal								Normal		Excellent	0	
End of Eighth Quarter																		
B						Discharged												
F		30 cc				Still under treatment												
J						Discharged						+50%		95	98.6	Improved	0	

One of the chief considerations in prostatectomy is to avoid injuring the verumontanum with its ejaculatory ducts, yet little importance had been attached after operation to the seminal vesicles and their ducts as possible sites of infection

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* Read before the Section on Urology at the Eightieth Annual Session of the American Medical Association Portland Ore July 12 1929

In the past few years, when possible, I have insisted at what I considered the opportune time, on a full physical examination of my prostatectomized patients, in addition to my own special investigation of the genitourinary tract, and have been surprised at the frequency with which seminal vesiculitis was encountered

I agree with Montague L Boyd,[1] who believes that seminal vesiculitis is a direct extension from the prostate bed, as four of the fifteen cases in the accompanying tables did not show any preoperative infection in the bladder, prostate or vesicles I realize, however, that the prostate bed and vesicles could be infected by urine that has become infected after operation

TABLE 2—*Data in Five Additional Cases*

Case	Residual Urine: Preoperative	Residual Urine: Postoperative	Preoperative Bladder	Laboratory Observations, Postoperative: Bladder Infection	Bladder Pus	Seminal Vesicles Infection	Seminal Vesicles Pus	Kidneys Infection	Kidneys Pus	Permanent Phenolsulphonphthalein Maximum: Preoperative Average 1st Hour	Preoperative Average 2d Hour	Postoperative Average 1st Hour	Postoperative Average 2d Hour	Average Temperature	General Condition	Epididymitis	Rectal Observations Palpable Vesicles Perivesiculitis
End of First Quarter																	
C	90 cc	20 cc	B coli	+++	++	+	+++	0	0	20%	25%	20%	25%	98 to 99	Poor	0	Definite in all
H	140 cc	10 cc	Streptococcus non hemolyticus	+++	++	++	+++	0	0	35%	30%	35%	20%			+ 4th day	
I	35 cc.	0	0	+++	++	++	+++	0	0	25%	25%	30%	20%			+ 7th day	
L	20 cc	0	0	++	++	++	++	0	0	35%	20%	30%	20%			0	
N	20 cc	0	0	++	++	+	++	0	0	30%	15%	80%	20%			0	
End of Second Quarter																	
C		20 cc		+++	++	+	+++	0	0			25%	25%	98 to 99	Improved	0	Less
H		0		+++	++	++	+++	0	0			Others about same				0	
I		0		+++	++	++	+++	0	0							0	
L		0		++	++	++	++	0	0							Slight induration	
N		0		++	++	+	++	0	0								
End of Third Quarter																	
C		20 cc		Slight improvement								25%	20%	98 to 99	Improved	0	0
H		0										Others same					
I		0															
L		0															
N		0															
End of Fourth Quarter																	
C		12 cc		++	++	+	+	Same				25%	20%	96 to 98 6	Improved	0	0
H		0		++	++	+	+										
I		0		++	+	+	0										
L		0		+	++	++	+										
N		0		+	++	++	+										
End of Fifth Quarter																	
C		12 cc		+	+	+	+	Same				Same		Normal	Improved	0	0
H		0		+	+	+	+							Normal			
I		0		0	+	+	+							Normal			
L		0		0	+	++	+							Normal			
N		0		0	+	++	+							98 to 98 6			
End of Sixth Quarter																	
C		12 cc		Same		0	+	0				Same				0	0
H		0															
I		0															
L		0															
N		0															
End of Seventh Quarter																	
C		12 cc		+	+	0	0	0						Normal	Improved	0	0
H		0		+	+	0	0										
I		0		0	+	0	0										
L		0		0	+	0	+										
N		0		0	+	0	+										
End of Eighth Quarter																	
C		0		+	+	0	+	0						Normal	Excellent	0	0
H		0		+	0	0	+										
I		0		+	0	0	0										
L		0		0	+	0	+										
N		0		0	+	0	+										

In this paper, based on fifteen cases representing approximately 25 per cent of the prostatectomized patients that I have been able to follow up in the last six years, I will show that responsibility *does not cease* when the incision is healed and voiding of urine is established After infection in the upper urinary tract and the possibility of obstruction to the flow of urine are eliminated, one may find that the patient is not improving satisfactorily The general physical examination in my patients was negative but my own investigation of the bladder and seminal vesicles revealed the following symptoms and observations The patients complained of suprapubic pain, perineal discomfort, testicular pain, vague joint and muscle pains, urinary frequency and, in some instances, painful erection occurring irregularly during the twenty-four hours Examination of the urine showed pus and infection Rectal examination elicited a sensitive prostate bed, sensitive indurated seminal vesicles with perivesiculitis The expressed secretion showed pus in varying degrees

The accompanying tables will serve to illustrate my observations They represent a practical compilation of data gathered from a follow-up study of fifteen cases over a period of six years, each case having been under observation for at least two years

ANALYSIS OF THE TABLES

A plus sign in the tables used in connection with the pus content represents twenty-five pus cells to the high dry field

Used in connection with infection, one plus sign means light growth, two plus signs, moderate growth, and three plus signs, heavy growth

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[1] Boyd M L Seminal Vesiculitis After Prostatectomy Due to Infection with Micrococcus Catarrhalis J Urol **10** 387 (Nov) 1923

Group 1 includes cases in which examination was done six weeks after the wound was healed and urination was restored The general physical examination was negative The urologic examination revealed seminal vesiculitis

Group 2 gave the same observations as in group 1, when examination was done seven weeks after closure

Group 3 showed the same condition as group 1 when examination was done nine weeks after closure

Residual Urine—The postoperative residual urine did not bear relation to the preoperative residual urine except when a large residual urine had existed for years, causing a permanent though slight loss of bladder tone F, in group 2, was an example of this

Laboratory Observations—In four of the fifteen cases there was sterile urine before operation After operation, cultures in all were positive The culture in eleven showed essentially the same organisms as were present previous to operation Specimens of urine taken direct from the kidney pelves were normal in all three groups From the tables it would seem that at the end of each quarter there was a gradual decrease in the pus content and infection in both the bladder urine and the secretion from the seminal vesicles This was not the case, however, as the pus content and infection showed many variations during the quarters, but the general tendency was toward decrease

The Permanent Phenolsulphonphthalein Maximum—Four of the fifteen patients were prepared for operation with indwelling catheters In the others that test was repeated frequently and a blood urea estimation was carried out at the same time It is interesting to note that in thirteen cases the good average preoperative maximum phenolsulphonphthalein percentage equaled the maximum postoperative phenolsulphonphthalein to the eighth quarter In the other two cases there was a fall in the postoperative phenolsulphonphthalein at first, which became higher than the preoperative phenolsulphonphthalein maximum later

Temperature—The temperature is typical of a low grade infection, slightly below or above normal some time during the day In two cases a slightly subnormal morning temperature still exists

Epididymitis—This occurred in two cases on the fourth and seventh days, respectively

TREATMENT

A bland, simple diet was prescribed Stimulating tonics were given with port wine or whisky to definite advantage

Acriflavine hydrochloride, salol hexylresorcinol and methenamine, administered by mouth, were the urinary antiseptics that gave the best results

Locally gentle stripping of the seminal vesicles once a week or as indicated, with instillations to the posterior urethra of mercurochrome-220 soluble, 1 to 3 per cent, mercurophen 1 in 5,000, or mild silver protein 10 per cent along with water or diathermic rectophore, was found the method of choice Autogenous vaccines and milk and other foreign proteins were also used to some advantage

I was unsuccessful in getting bacteriophage in any of these cases

SUMMARY

N P Rathbun[2] quotes ten cases in which prostatectomy was done Nine showed seminal vesiculitis following operation, a total of 90 per cent I found seminal vesiculitis in 25 per cent of sixty carefully followed cases, which I believe warrants the statement that the surgeon's responsibility does not cease when the closure of the wound has taken place and voiding has been established

CONCLUSIONS

The study of these cases has convinced me that recognition and proper treatment of seminal vesiculitis occurring after prostatectomy is just as important a factor in the comfort and longevity of the patient, as preoperative care is to a safe prostatectomy

384 Post Street

POSTOPERATIVE COMPLICATIONS OF PROSTATECTOMY *

A H PEACOCK, M D

SEATTLE

Science judgment, experience and surgical skill are the four guardians to safety and success in prostatectomy Adversity and misfortune are often master teachers and from them we learn much that is valuable I believe that the urologist has an obligation toward the general surgeon What knowledge he has gained should be passed on to him In the open staff and private hospitals, only a small part of the urologic cases admitted are handled exclusively by urologists In the smaller towns and cities this situation is even more prevalent

What interests the surgeon most in the operation of prostatectomy? Is it the report of 100 or 200 consecutive cases without a death? It is the postoperative complications which hold his attention He must manage the average elderly man partly broken in health After careful preoperative preparation, as decompression of the urinary bladder, stabilization of the cardiovascular system, improving the renal function, obtaining blood counts and chemical estimations of the blood, improving the nutrition, watching a smoldering respiratory infection, and getting the old gentleman into the proper frame of mind, then last comes the surgical remedy

The observation notes in this paper are made from a series of 117 cases in private practice The working conditions have not been ideal because of the lack of organized clinics and urologic wards In studying cases for the complications and end-results, one should strive for the truth One wants to see if a mistake in judgment has been made and how to avoid it next time Inaccurate reports do not deceive any one but the writer

Complications may be divided into immediate and delayed The immediate are those occurring within forty-eight hours and the delayed after this period Under immediate complications are renal failure, cardiac failure, thrombosis and hemorrhage

RENAL FAILURE

In spite of renal function and the chemical condition of the blood within the safety zone, this complication should be prepared for in all cases Out of forty-two deaths in 995 cases at the Mayo Clinic, Hunt states that 25 per cent were due to uremia It is a good practice to digitalize the prostatic patient before the operation and to give fluids intravenously immediately following. Mental indifference, drowsiness and failing urinary output require prompt remedies. Gradual decompression, preliminary drainage, and cystotomy have lowered the mortality greatly. Many men past 60 years of age have hypertension and a trace of albumin. This should always be a warning. Age has brought about changes in the kidney, as arteriosclerosis and degeneration of the epithelium of the tubules. Under ordinary conditions the kidney function is sufficient but when put on a strain, as violent fluctuations of the blood pressure or shock, renal excretion is impaired, with increasing blood retention of urea nitrogen, creatinine and chlorides. Symptoms of toxemia appear, restlessness, diminished urinary output, drowsiness, nausea and vomiting. Out of eight deaths in this series of 117 cases, 37.7 per cent were due to renal failure.

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[2] Rathbun N P Seminal Vesiculitis as a Complication of Prostatism and Prostatectomy Surg Gynec Obst **10** 214 (Feb) 1925

* Read before the Section on Urology at the Eightieth Annual Session of the American Medical Association Portland Ore July 12, 1929

Case 1.—F. F. R., a man aged 70, an attorney, had symptoms of prostatic obstruction for the past seventeen years and used a catheter for a long time. Hematuria developed, making catheterizing difficult. A diabetes mellitus had been controlled with insulin.

The patient's height was 5 feet 11 inches (180 cm.) and he weighed 200 pounds (91 Kg.). His blood pressure was 180 systolic and 120 diastolic. Chemical analysis of the blood showed sugar, 130.4 mg.; creatinine, 2.22 mg., and urea nitrogen 12.14 mg. The phenolsulphonphthalein output was 60 per cent in two hours.

Urinalysis revealed a specific gravity of 1.018, a trace of albumin, faint trace of sugar, many pus cells, numerous red blood cells and colon-like bacilli.

Sept. 4, 1928, an indwelling catheter was used and a right sided epididymitis developed. The catheter was not a success, so on September 8 a suprapubic cystotomy was performed under local anesthesia. The epididymitis later progressed to suppuration before it subsided.

September 28, a suprapubic prostatectomy was done under sacral anesthesia. In spite of diuretic drugs and intravenous fluids, the urinary output fell off rapidly. Forty-eight hours afterward convulsions set in and death followed.

The explanation of this death was severe long standing urinary infection and a chronic nephritis. The patient might possibly have been saved had a longer period of drainage been given him. However, he was extremely hard to manage, nervous, impatient and headstrong.

Case 2.—W. S., a man, aged 90, a farmer for years, had prostatic obstruction until complete retention occurred. Catheterization could not be effected, so a suprapubic cystotomy was done, Oct. 14, 1928. He was also afflicted with a distressing bronchitis of long standing.

His height was 5 feet 8½ inches (173 cm.) and his weight 115 pounds (52 Kg.) at the time of examination, his normal weight was 165 pounds (75 Kg.). His blood pressure was 150 systolic and 90 diastolic. Chemical analysis of the blood revealed urea nitrogen 28 mg., creatinine 2 mg. and nonprotein nitrogen 55.5 mg. The phenolsulphonphthalein for the first hour was 15 per cent, the second hour, 20 per cent. Urinalysis showed a trace of albumin with many pus cells and bacteria.

October 30, a suprapubic prostatectomy was done under sacral anesthesia. On the sixth day the patient was listless and could swallow only with difficulty. Breathing was labored from mucus in the bronchi. He gently slipped into unconsciousness and died on the fourteenth postoperative day. Shock and renal failure caused his death.

Case 3.—W. U., a man aged 72, a laborer, suffered with a severe backache for months. His frequent urination he accepted as part of his age. The attending physician found a distended bladder and relieved him of 2 quarts (liters) of urine. Since then a catheter had been necessary to relieve him.

His height was 5 feet 8 inches (172.7 cm.), his weight 120 pounds (54 Kg.) at examination, his normal weight was 165 pounds (75 Kg.). He was a slender, elderly man with a morose pessimistic disposition. Life had been a failure and disappointment to him. He had worked unceasingly all his life, only to break down physically and find himself without friends and financial aid. I mention this, as I had this mental attitude to combat throughout his illness. The mental attitude of the patient is a tremendous factor in his recovery. For ten days this patient refused to eat, so that forced feeding and intravenous dextrose solution was employed until he got tired of it and ate as he should have.

The blood pressure was 90 systolic and 60 diastolic. Chemical analysis of the blood showed urea nitrogen, 60 mg., and creatinine, 3.31 mg. After six weeks with catheter drainage, the urea nitrogen dropped to 30 mg. and the creatinine to 1.8 mg. The phenolsulphonphthalein output for the first hour was 0 per cent, the second hour, 5 per cent, this rose to 17.5 per cent. This delayed output for the first hour is significant of serious kidney impairment. The patient seemed to have reached his maximum improvement.

A prostatectomy was done under gas anesthesia Nov. 12, 1924, after two months' drainage. He seemed to do well after his operation except for his nourishment, as he again refused to eat. He lapsed into unconsciousness on the fourteenth postoperative day and died, apparently uremic.

CARDIAC FAILURE

In cardiac failure one is again confronted with a vital organ that age has impaired. The heart has faithfully performed its muscular contractions for sixty, seventy or eighty years, under all conditions and experiences, joy and sorrow, muscular exertion and rest, health and illness. It is well known that the heart will not stand the strain at 60 years that it will at 20, for myocardial degenerations occur. All athletes will attest to this fact. Past middle life, the heart is always an uncertain factor. One patient of 60 years, I remember, had been hospitalized for prostatectomy apparently in good condition, he died suddenly of coronary thrombosis awaiting operation. In this series, 25 per cent of the deaths were due to cardiac disease.

Case 4.—G. M. H., a surgeon, aged 65, for two years while attending to his large surgical and general practice catheterized himself four times a day. Infection of the bladder resulted. He became so irritable and distressed that prostatectomy was requested April 26, 1927. One year previous he collapsed in the operating room and was confined to bed for eight weeks with a myocarditis.

The patient's height was 5 feet 11 inches (180 cm.), his weight 200 pounds (91 Kg.). The blood pressure was 205 systolic and 105 diastolic. Chemical analysis of the blood showed urea nitrogen, 12.5 mg.; nonprotein nitrogen, 27.2 mg.; chlorides, 462 mg., and creatinine 1.3 mg. The phenolsulphonphthalein output for the first hour was 20 per cent, the second hour, 12 per cent. The urine showed a trace of albumin, occasional hyaline casts and the presence of staphylococci.

This patient was dictatorial and domineering, he knew just what he wanted and when it was to be done. An immediate one-stage prostatectomy was insisted on. A suprapubic operation under gas anesthesia was done. The patient left the table in good condition. Two hours later, his pulse suddenly became imperceptible, he thrashed around the bed, Cheyne-Stokes breathing developed and he died four hours after the operation. Death was due to coronary thrombosis.

Two mistakes were made: a two stage operation was clearly indicated and sacral anesthesia should have been employed. Irrespective of the patient, one should never be persuaded into acting against one's judgment. It would be far better to refuse one's services altogether.

Case 5.—A. B., aged 79, an investment broker, sought relief April 18, 1927, for retention of urine. Catheterization had been necessary for two months. For years he had suffered with hypertension and myocardial disease. Recently he remained in bed feeling too weak to sit up. He was 30 pounds (14 Kg.) underweight.

The patient's height was 5 feet 10 inches (185 cm.), his weight was 110 pounds (50 Kg.). The blood pressure was 170 systolic and 90 diastolic. The heart was markedly enlarged and auscultation showed an auricular fibrillation Chemical analysis of the blood showed urea, 63 9 mg, and urea nitrogen, 29 9 mg A phenolsulphonphthalein test gave 40 per cent for the first hour and 25 per cent for the second

An indwelling catheter was used On his seventeenth hospital day the patient developed a slow cerebral hemorrhage which clouded his speech this cleared up in five days On the thirtieth hospital day a suprapubic cystotomy was done under local anesthesia He developed a slight cough and rales were heard over the bronchi This cleared up under diathermy Three weeks after the cystotomy the prostate was enucleated under ethylene gas anesthesia Forty eight hours later he suffered a cerebral hemorrhage, lapsed into unconsciousness and died three days later This patient was handled most carefully Death would probably have occurred in any event We sometimes think we can do the impossible.

Combined cardiac and renal disease is a problem to all surgery The mortality is always high

Case 6—J G M aged 57, a cook, had been ill with mitral disease for years several times almost dying This condition was secondary to inflammatory rheumatism For a year he had great difficulty in voiding, resulting in complete retention He was catheterized after considerable effort

At examination June 17, 1927 the patient's breathing was labored and he had moderate cyanosis His height was 5 feet 7 inches (170 cm) his weight 200 pounds (91 Kg) The blood pressure was 160 systolic and 80 diastolic the pulse rate was 104 There was edema of the legs and ankles The urine showed a heavy ring of albumin and hyaline casts The heart was somewhat enlarged, very irregular and produced a loud mitral murmur There was a suprapubic dulness Chemical analysis of the blood showed urea nitrogen, 18 mg urea 32.52 mg., and creatinine 1 5 mg The phenolsulphonphthalein test gave 5 per cent the first hour and 22.5 per cent the second hour As the catheter was so painful a suprapubic cystotomy was done under local anesthesia. The pulse measured from 130 to 145 beats and the respiration 38 to 40 for a week The patient s condition seemed so improved that a prostatectomy was done one month later under sacral anesthesia Two weeks later he was sent home to convalesce, the wound was almost healed Five days afterward he became irrational, when coma developed followed by death two days later

This patient, at all times was a poor risk His retention, the great difficulty and pain from the catheter decided us to resort to surgery There seemed to be no other solution, as the patient complained bitterly of pain

Urinary infections play an important role in these complications Retention of urine, frequent catheterization under unfavorable conditions, chronic pulmonary infections, intestinal stasis and infected teeth are a menace In Hunt's report, 16 5 per cent of the deaths were due to general sepsis

In the present series of 117 cases, epididymitis occurred in three cases before operation and in ten cases after operation It was a distressing condition but not fatal This complication occurred in 11 per cent of cases Many advocate preliminary vasectomy In 20 per cent of 1,049 cases reported by Young, epididymitis developed

Pyelitis occurred in two cases They responded to treatment readily

Severe cystitis was noted in seventeen cases, or 14 5 per cent These infections were usually from *Bacillus coli* and staphylococci They cleared up after drainage but usually caused a delay in the healing of the wound

Pyonephrosis occurred in two cases and later nephrectomy was done In cystoscopy of the prostatic patient I do not always catheterize the ureters I think that if I did valuable information might be obtained, especially when there is an infection

The pulmonary complications are lobar pneumonia, bronchopneumonia, chronic bronchitis lung abscess and embolism Pulmonary complications occurred in 11 11 per cent of patients as follows pneumonia, five, lung abscess, three, embolism, one, and chronic bronchitis four Seven of these had sacral anesthesia and six had general anesthesia

Case 7—S S, aged 80, a farmer, suffered for seven years with difficult urination, nocturia and hematuria He had influenza and pneumonia in 1918

At examination, April 5, 1924 he weighed 165 pounds (75 Kg) his height was 5 feet 8 inches The blood pressure was 150 systolic and 90 diastolic Chemical analysis of the blood showed urea, 57 6 mg, and creatinine, 1 9 mg

After preliminary catheter drainage, a one step suprapubic prostatectomy was done under gas anesthesia After thirty-six hours the respirations went to 50 and the pulse to 120 Breathing was difficult The next day there were all the signs of a pneumonia He died on the fifth postoperative day Sacral or spinal anesthesia which he refused would probably have saved this man Local anesthesia has ushered in a new era for prostatectomy

Outline of Cases

Total cases				117
1 Suprapubic			111	
(a) One stage		60		
(b) Two stage		54		
2 Perineal			3	
Surgical deaths				8
Mortality			6.8%	
Age incidence				
Oldest	90			
Youngest	44			
Average	69 4			
Complications				
1 Immediate				23
2 Late				35
A Pulmonary				13
1 Pneumonia	5 } Anesthetic used			
2 Lung abscess	3 } sacral 2 general 6			
3 Embolism	1			
4 Chronic bronchitis	4			
B Cardiac				10
C Urinary tract infections				81
1 Epididymitis				
Preoperative		3		
Postoperative		10		
2 Pyelitis		2		
3 Cystitis		17		
4 Pyelonephritis		2		
D Renal insufficiency				6
F Hemorrhage				5
F Persistent sinus				2
G Cerebral embolism				2
H Severe hemorrhage				2

Case 8—N D H a gardener, aged 76 suffered with urinary obstruction until it became complete He catheterized himself and shortly developed an epididymitis He was admitted to the hospital for retention April 13, 1928

His height was 5 feet 8 inches (173 cm) and his weight 145 pounds (66 Kg) The blood pressure was 135 systolic and 70 diastolic The temperature was 100 F at noon Physical signs of locomotor ataxia were present. The blood Wassermann reaction was negative. Chemical analysis of the blood showed urea, 75 mg and creatinine, 1 4 mg The phenolsulphonphthalein test the first hour was 10 per cent, the second hour 5 per cent

After eight days of drainage a suprapubic cystotomy under local anesthesia was done. Eight days later the prostate was enucleated under sacral anesthesia After two days the patient had a chill a temperature of 103 F, cough and dark sputum On the seventeenth postoperative day it was possible to make a diagnosis of pulmonary abscess of the right upper lobe Death occurred on the twenty-fifth postoperative day The wound had healed This patient had the handicap of an infection to start with

COMMENT

Postoperative hemorrhage has always been a threat to prostatectomy, either immediate or secondary Immediate hemorrhage occurred in two cases, in both of which Pilcher bags were inserted In each case the bag proved too small The bag was removed, a sound then passed into the urethra and two inch gauze packing used to fill the prostatic cavity Once certain of a hemorrhage, one should not lose any time in removing everything, to inspect the prostatic bed and pack tightly

Secondary hemorrhages occurred in three cases These came on at the end of ten days or two weeks, they were not as severe as the primary, being easily controlled by packing They were the result of an erosion of a vessel in the wound Often large vessels in the bladder wall are the ones involved Direct vision prostatectomy has reduced hemorrhage The mortality of these 117 cases was 6 8 per cent and each death has been reported in brief with the hope that others may profit by my errors of judgment

SUMMARY

1 The study of the postoperative complications of prostatectomy is an aid in further reducing the mortality of this operation

2 The chief complications are renal failure, cardiac failure, pulmonary infections, urinary infections and embolism

3 These complications can be handled better by giving a longer time to preliminary drainage and preparation

4 The prostatic patient often has multiple pathologic changes and should be studied thoroughly

5 In 117 cases, the mortality was 6 8 per cent

6 Of the deaths, 37 7 per cent were due to renal failure, 25 per cent to cardiac failure and 25 per cent to pulmonary disease

1215 Fourth Avenue

ABSTRACT OF DISCUSSION

ON PAPERS OF DRS PLAYER AND PEACOCK

Dr Floyd F Hatch Salt Lake City One point not fully dwelt on is the control of hemorrhage The suturing of the bladder floor by the method of Hunt and Sir John Walker or after the manner of Lower is one of the best methods of reducing the frequency of this complication In addition we can add the bag if necessary If we do this we can feel much more at ease Another point concerns the complication of pulmonary embolism I think that the chest irritation and the various symptoms that are minor in the chest which follow inhalation of the gas ether or whatever we use are often due to the embolic fragments from the wound that get up to the lung area Another thing is that the fatal embolisms are rather common in prostatectomies I think that extreme gentleness in a procedure that at times must be rather rough is well worth bearing in mind

Dr John R Caulk St Louis It is a current conception that it is difficult to expose the neck of the bladder in a two-stage prostatectomy For two years I have been opening the wound at the point of incision exposing the bladder neck thoroughly grasping the orifice and pulling the prostate to the operator in order to lessen trauma from manipulation and to simplify enucleation I have modified the Judd retractor by lengthening it one half inch and tapering it so as to make it enter the bladder opening In this way one can secure perfect exposure of the bladder neck, trim away tags which often cannot be palpated and also clamp and ligate any bleeding arteries This procedure takes longer than the average blind second stage prostatectomy It shortens convalescence lessens complications and certainly minimizes mortality

Dr. George W Hartman San Francisco It may be of interest to mention two cases in which the end-results had the patients been operated on would have been regarded as complications' following prostatectomy The first case was that of a man with carcinoma of the prostate, without metastasis but with fairly advanced Parkinson s disease Because of the patient s inability to handle a catheter, all tests and functions being satisfactory, we decided to remove the prostate While being prepared for operation the patient died A careful necropsy did not disclose any special cause for death I do not believe that scopolamine, which I allowed the intern to administer for the successful control of the parkinsonian tremor, was responsible for the death Had this patient been operated on, I am sure that death would have been attributed to the surgery The other patient had the largest prostate I have ever seen He had complete retention, low function and a heart so bad that the cardiologist reported his condition as utterly hopeless By catheter drainage the patient improved so much that he was able to return to his work as a butcher Under observation he did so well that we began to contemplate prostatectomy One afternoon he complained of a severe pain in his thigh He was admitted to the medical service of the hospital Shortly afterward, he developed a swelling of the right ankle which was incised and found to contain pus Blood culture showed the presence of colon bacilli At necropsy a large right sided psoas abscess was found, which on further search was traced to a small intraprostatic one. At the 1928 meeting of the western branch of the American Urological Association, the discussion of postoperative enemas almost convinced me that there was no accompanying danger Recently a death due to embolus following an enema was reported by C P Henry For this reason I have again forbidden the use of enemas A good clue to the patient s progress, preoperative or postoperative, is the condition of the tongue, which should be watched closely Varying degrees of dryness, and thickness of the coating, changing from white to yellow and finally to brown, give a close index of the patient's status Noted early enough it can frequently be relieved by forcing fluids through any channel available

Dr. Bransford Lewis St Louis In my desire to fortify against hemorrhage in connection with prostatectomy about five or six years ago, I thought it would be well to anticipate hemorrhage by giving one of the blood-clotting agents, but I had the misfortune to have a bad result The only time I ever had emboli form in prostatic surgery was then I had three cases within a few days Two of the patients died and the third had a paralytic condition of the left arm I stopped using these agents and have had no emboli since A little point that to me has seemed helpful is that when we have the internist examine the heart which we do preliminary to any prostatic work, and he says there is no objection to operating, we give digitalis for two or three days I have had reason to regret not giving it in some cases, and I deem it a valuable adjunct to the preoperative care

Dr Alexander H Peacock, Seattle We are indeed indebted to Dr Player for calling our attention again to the chronic infection of the seminal vesicles I feel that these chronic vesicle infections account for most of the cases of epididymitis and also for many cases in which the urinary infection does not clear up after prostatectomy

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Single Symptoms and Faulty Diagnosis—With the unwary and inexperienced the significance of a single symptom may be overstressed and lead to a faulty or incomplete diagnosis Palpitation of an abnormal pulse caused by indigestion or excessive smoking may suggest heart disease and lead to the inappropriate prescription of digitalis Diarrhea may be a simple matter yielding readily to a chalk and opium mixture but, on the other hand the looseness may be due to irritation of the bowel from the presence of scybala, the fluid motion being Nature s effort to wash them away Treatment in such a case is by laxatives and enemas rather than by astringents Again this symptom may occur in cancer of the rectum I have met with two such cases within a month, in one of which —a cardiac patient—there was occasional bleeding wrongly attributed to piles Digital examination revealed cancer of the rectum in both On one occasion when several visitors accompanied me around the wards in hospital, one of the patients had in addition to other symptoms, dilatation of the left pupil Cerebral thrombosis was among many explanations suggested, but, as a matter of fact the right eye was an artificial one and the left pupil dilated in response to the darkness of a November fog!—Chapman, C W Pitfalls in General Practice, *Practitioner* October, 1929

A CLASSIFICATION OF CHRONIC IDIOPATHIC SECONDARY ANEMIA

WITH ESPECIAL REFERENCE TO THE MORPHOLOGY OF THE BLOOD *

CHARLES H WATKINS, M D
ROCHESTER, MINN

Chronic secondary anemia may be divided into three general groups a type due to hemorrhage, a type due to some organic lesion and, after all known causes of anemia have been excluded, a type which it has been necessary to classify as idiopathic secondary anemia The last-named type has been difficult to treat in comparison with the first two types, which respond as a rule following removal of the causative factor

Because of difficulty in the treatment of the forms of idiopathic secondary anemia, it seemed that a study of the morphology of the blood in these cases might throw some light on the condition of the hematopoietic organs, and thus might enable treatment to be directed toward restoring normal hematopoietic function

Before discussing the morphologic blood picture in idiopathic secondary anemia it is well to list the more characteristic features of regeneration and degeneration of the blood that appear in idiopathic secondary anemia The chief features of regeneration are (1) anisocytosis, variation in the size of erythrocytes, with a liberal number of macrocytes, (2) polychromatophilia, in which the basophilic spongioplasm has not been entirely replaced by hemoglobin, depending on the degree of the regeneration, (3) reticulocytes, immature erythrocytes in which the basophilic spongioplasm, not yet replaced by hemoglobin, is stained by a vital dye, (4) normoblasts, nucleated erythrocytes of the definitive series, and (5) multiple Howell-Jolly bodies pink-staining rounded bodies in the erythrocytes, representing the remains of the parachromatin of the nucleus after the chromatin has been dissolved in or extruded from the cell

Besides these features of regeneration, the myeloid leukocytes show a tendency toward decrease in lobulation with less condensation of chromatin, the so-called shift to the left

The outstanding features of normal degeneration are (1) anisocytosis, variation in the size of erythrocytes with a high proportion of microcytes, (2) hypochromasia, decrease in hemoglobin in the erythrocytes so that the cell is uniformly pale, and (3) anochromasia, piling up of the hemoglobin in a narrow ring around the peripheral portion of the cell, leaving a wide, colorless central zone Besides these features of degeneration there is a tendency toward a shift to the right of the myeloid leukocytes, an increase in the number of lobules of the nucleus, with moderate condensation of the chromatin In this connection, the morphology of the "toxic neutrophil" and the "pernicious anemia neutrophil" should be differentiated The toxic neutrophil shows a shift to the left averaging two distinct lobes, although the individual lobes may be serrated, giving a multilobed appearance As a rule there are heavy knoblike projections on the lobes and one lobe is larger than the other The neutrophilic granulation is more deeply staining and the individual granules are larger than normal The toxic monocyte has a sharp indentation in the nucleus, with condensation of the chromatin, and a coarse, heavy, azurophilic granulation The "pernicious anemia neutrophil," on the contrary, is shifted to the right, having an average of more than five lobes in the nucleus, the lobes are thinner and smaller than normal and there is distinct stranding between individual lobes The cell is generally larger than the normal cell

Chronic idiopathic secondary anemia may be subdivided into four general types according to the morphologic blood picture

The first type usually occurs in young persons, and primarily in females The proportion of hemoglobin is moderately reduced, averaging approximately 50 to 55 per cent, the number of erythrocytes is slightly reduced, averaging about 4,000,000 in each cubic millimeter, and giving a color index of 0 5 to 0 6 The reticulocytes are generally normal or slightly increased in number Morphologically, the erythrocytes are characterized by marked anochromasia, moderate anisocytosis, slight hypochromasia, slight polychromatophilia and practically no other change The marked anochromasia is the outstanding feature of the blood The leukocytes are essentially normal Because of the similarity of the blood picture to chlorosis this has been termed the chlorotic type It has been assumed that this type of anemia is due to dietary deficiency, to poor hygienic conditions or to a combination of the two Rapid improvement in these cases has been obtained by the use of a well balanced diet, iron in therapeutic doses, fresh air and sunshine

The second type of secondary anemia is characterized by a proportionate reduction in the hemoglobin and the erythrocyte count, so that the color index is approximately 1 The number of reticulocytes is decreased or they are absent entirely, and the leukocyte count is slightly reduced A characteristic of the erythrocytes, for the most part, is normochromasia, but there is usually associated slight anochromasia, moderate anisocytosis without macrocytosis or polychromatophilia The morphology of the granular leukocytes is probably the most characteristic feature The neutrophils are slightly decreased in size There is increased lobulation of the nucleus, the majority of the cells have a nucleus of four or five lobes The lobes are of normal contour, without thinning or stranding, and the granules are pale and irregularly distributed in the cytoplasm It is important not to confuse this cell with the pernicious anemia neutrophil The monocytes show a tendency toward lobulation of the nucleus, so that most of these cells contain a saddle-shaped nucleus The azurophilic granulation is heavier, as a rule, than in the normal monocyte The other leukocytes are essentially normal There is generally slight relative lymphocytosis The blood picture, as a whole, would be described as "aged," and it is evident that there is a decrease in activity of the bone marrow, probably a predisposition on the part of the patient to dysfunction of the bone marrow Because of this, it has been termed the semiaplastic type of secondary anemia Certain toxic and infectious processes often produce a similar type of anemia, which may be identified readily by the toxic changes in the neutrophils and monocytes

The treatment is directed toward stimulation of activity of the bone marrow This may be accomplished by various methods transfusion, intramuscular injec-

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* From the Division of Medicine the Mayo Clinic

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tions of whole blood,[1] or the use of an experimental extract of bone marrow with which I have had very good results In many instances it will be found that the increase in erythrocytes is more rapid than the increase in hemoglobin, so that the color index, which was approximately 1, is converted to an index of less than 1 Because of this, a diet high in iron should be included in the treatment

The third type of chronic secondary anemia occurs chiefly in women between the ages of 30 and 50 As a rule, it is insidious in onset By the time the patient seeks advice, the condition usually has been present for several years, even as long as ten or fifteen years, and has failed to respond to the usual treatment of secondary anemia There may or may not be associated achlorhydria The clinical syndrome has long been recognized by the physician, and it often has been considered as being indicative of an early stage of pernicious anemia However, during the observation of many of these cases over long periods of time, there has been no change to pernicious anemia The condition of the blood is usually characteristic The proportion of hemoglobin is reduced, averaging from 45 to 50 per cent, whereas the erythrocyte count remains relatively high The number of reticulocytes usually is slightly increased, and the leukocyte count is normal Morphologically, the outstanding characteristic of the blood picture is the marked hypochromasia of the erythrocytes, the cells having a pale, washed-out appearance There is also slight anochromasia of some of the erythrocytes, and slight polychromatophilia The leukocytes are essentially normal morphologically In this type, the function of the bone marrow appears to be normal, the dysfunction apparently is in the synthesis of hemoglobin, consequently it has been called the hemoglobin dysfunction type Because of the dysfunction it was assumed that the fetal liver of calves, obtained at the time of the greatest hematopoietic function of the liver, might contain a substance that would stimulate synthesis of hemoglobin[2] A powder was made from fetal livers and was used, in a dosage corresponding to 300 Gm of the raw fetal liver a day, in the treatment of this type of anemia There was marked response to its use in practically all of the cases In those in which toxic neutrophils and toxic monocytes have been found, there is practically no response until the causative factors have been eliminated Apparently, after the hemoglobin has increased to its normal value, it has a tendency to remain at that level without further treatment, at least for an appreciable period of time

The fourth type of secondary anemia has been found only in young persons whose parents or other near relatives have had pernicious anemia The erythrocyte count is slightly reduced and the hemoglobin is moderately reduced, so that the color index is less than 1 There is moderate hypochromasia and moderate anochromasia of erythrocytes There is generally slight anisocytosis with but slight macrocytosis The most characteristic feature of the blood picture is the morphology of the neutrophils The number of lobes of the neutrophilic nucleus is increased, averaging from five to seven, and there is a tendency toward definite thinning and stranding of the individual lobes, as in the true pernicious anemia neutrophil Because of the familial history of pernicious anemia, and since the pernicious anemia neutrophil was found in the blood, this has been called the potential pernicious anemia type of secondary anemia and small amounts of raw liver or liver extract were given to these patients An exceedingly rapid increase in hemoglobin and erythrocytes occurred, so that in a period of approximately ten days the blood count had returned to normal

SUMMARY

The so-called idiopathic types of secondary anemia have been divided into four general groups Although the majority of such types of anemia will fall into one of these groups, the various blood pictures may be seen in varying degree and combinations in all types of secondary anemia, and only certain of them may be definitely classified A large series of cases has been studied This paper is presented as an outline of the subject It is probable that with further study a more inclusive classification may be made

ABSTRACT OF DISCUSSION

Dr. C B Wright, Minneapolis After seeing Dr Watkins' work and being helped by his suggestions in obscure cases, I became interested and put him to the following test I selected cases of benign anemia well known to me, in all of which I had tried the routine therapy of iron, heliotherapy, a diet of liver, and so on, without succeeding in getting the blood quite up to normal or in preventing rather rapid and unexplainable drops I submitted blood slides of these persons to Dr Watkins without any clinical report, and then treated them according to his recommendation The two cases which I treated with fetal liver were of long standing Following treatment, the hemoglobin rose to a normal level and remained there even several months after discontinuance of the treatment. In two cases of a mildly aplastic type, treated with bone marrow and spleen extract, the results were equally satisfactory, and in one case of a pseudopernicious type, a patient whose brother had died of pernicious anemia, and whose neutrophils showed pernicious anemia characteristics according to Watkins, associated with a high color index but normal gastric acidity, the blood count promptly increased on calf's liver extracts It has been observed before that some cases of secondary anemia would respond to liver extracts, but only the exceptional cases The results certainly were of interest. Dr Watkins does not think for a moment, I am sure, that he has said the last word in this field Whether his future work may undergo modification or not, there is no question in my mind that the time has come when reports of blood technicians should not be considered sufficient Obscure cases of anemia demand the study of highly trained blood morphologists working with the very best microscopes

Dr H B Sweetser, Jr, Minneapolis Is there any connection between the work we heard about this morning, with copper and iron, and this kind of anemia? Also, how does one get fetal liver? We have a patient in our office whose condition is similar to one of these types, and we have tried to get some fetal liver but have failed

Dr Rudolph C Logefeil, Minneapolis The average clinician even the internist, does not pay much attention to secondary anemia other than to determine that the patient has a reduction in the red cell count and hemoglobin and to distinguish whether or not he is dealing with primary or secondary anemia That is usually as far as it is carried If it is a primary anemia, the usual Minot-Murphy treatment is instituted while if it is secondary, effort is made to determine the cause, if possible, which should always be done, and then the usual diet with iron is given One of the most important things Dr Watkins has brought out, other than his classification and method by which we can separate these secondary anemias, are his suggestions as to treatment He has shown that something can be accomplished along this line, and it ought to stimulate each of us to make a more careful study and classification of secondary anemias and of the results from his suggested treatment. Since hearing his first talk I have had a great deal more interest in secondary anemia, and instead of depending on the reports of laboratories I have taken the time to use the microscope myself and to try to study some of these secondary anemias. It has been a source of great pleasure and has resulted in increased success in treating this common condition. I feel that Dr. Watkins should be complimented on his work and I hope he continues his studies along this line.

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[1] Morawitz P Einige neure Anschauungen uber Blutregeneration Ergbn d. inn Med u Kinderh 11 277 323 1913 Ritz, H Studien über Blutregeneration bei experimentellen Anamien Folio haemat. 8 186-193 1909

[2] Berglund Hilding Watkins C. H and Johnson Richard Rapid Stimulation of Hemoglobin Synthesis in Secondary Anemias After Feeding Fetal Calf s Liver Proc. Soc Exper Biol & Med 26 814-817 (June) 1928

DR. C. H. WATKINS, Rochester, Minn.: In reply to Dr. Sweetser's question regarding the use of copper and iron in secondary anemia, it is probable that those types of anemia which respond to copper and iron fall into the first group, the chlorotic type of secondary anemia. No analysis has been made as yet of the various elements of the fetal liver, and so I am not able to state at this time the amounts of copper and iron in it. The fact that the types of anemia in which the fetal liver was first used had not previously responded to the usual forms of treatment of secondary anemia leads to the assumption that there must be some other substance in the liver which brings about the response. As the use of the fetal liver is still experimental, we are making it at the Mayo Clinic in limited quantities.

ARGYROSIS OF THE CONJUNCTIVA

ITS SUCCESSFUL TREATMENT *

M. F. WEYMANN, M.D.
LOS ANGELES

Argyrosis of the conjunctiva is not an uncommon condition and usually follows prolonged local medication with silver nitrate or colloidal silver compounds, principally mild silver protein. Up to the present time there has been no successful method of removing the objectionable color which is present in argyrosis of the conjunctiva.

In conjunctival argyrosis there is a precipitate of silver albuminate and of silver oxide in the subconjunctival tissue. Fuchs[1] shows that the epithelium is unaffected but that the elastic fibers appear to contain brown punctate granules and there are a few extrafibrillar granules. He states that the silver is present as an organic compound. Gettler, Rhoads and Weiss[2] describe the silver particles in the skin as being found in the elastic fibers and the collagen fibers but none are intracellular. It would seem that as the conjunctiva is exposed to light, in the same manner as is a photographic plate, some of the silver might exist as metallic silver itself.

The presence in the tissue of these silver granules does not cause a reaction and is obnoxious only because of the gray brown color of the conjunctiva, particularly of the bulbar conjunctiva. Most of the severe cases of argyrosis I have seen have been caused by prolonged instillation of mild silver protein, probably because this heavy fluid tends to remain in the conjunctival sac for a long period of time and is slowly absorbed.

Recently Stillians and Lawless[3] have attempted to remove the pigmentation in argyrosis of the skin with some success. They have tried a reducing solution used in photography for reducing overexposed and overdeveloped plates and films. The usual photographic reducing solution, consisting of sodium thiosulphate and potassium ferricyanide, contains about one part of the latter to thirty-six parts of the former. But in their case they wanted as rapid a reduction as possible, so they finally used a solution consisting of equal parts of 12 per cent sodium thiosulphate solution and 2 per cent potassium ferricyanide solution. They tested the toxicity of ferricyanide on rabbits and found that one twenty-five thousandth of the body weight given intravenously did not cause any symptoms. Thus the average person weighing 160 pounds (73 Kg.) could stand at least 2 Gm. intravenously without danger. As the drug is rapidly oxidized in the lungs, the danger is lessened when absorption is slow as in intradermal or subconjunctival injection.

Stillians and Lawless found that they were able to remove the pigment of argyrosis in the skin by intradermal injection of the solution mentioned, but that the bleaching occurred directly only at the site at which the solution was injected. This made the treatment of large areas very difficult, but possible.

To test the reducing power of their solution, I applied it to an exposed and developed photographic plate. The 12 per cent thiosulphate solution alone had a slight reducing effect in two minutes; the 2 per cent ferricyanide had more of an effect, but the combination of equal parts of the two had a very marked action in dissolving the silver film on the plate. On the plate a solution of two parts of the ferricyanide to one of the thiosulphate had about the same effect as the solution containing equal parts, although clinically I obtained a more marked effect with the latter solution. Sodium thiosulphate instillations in the treatment of argyrosis have been suggested by Zur Nedden, but in two cases reported by Terlinck,[4] in which this method was used, very little removal of pigment was found. Stillians and Lawless tried both sodium thiosulphate and methenamine intravenously in their case of skin argyrosis without result. As they had shown that the thiosulphate-ferricyanide mixture caused little irritation when injected into the skin, I determined to try it on a patient with conjunctival argyrosis.

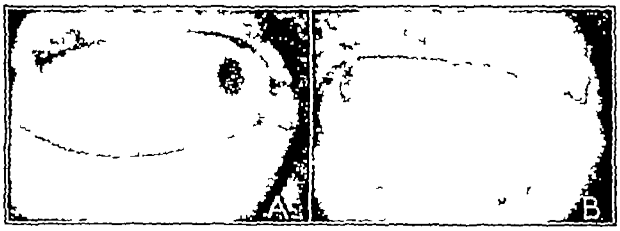

Fig. 1.—*A*, untreated eye; *B*, left eye after treatment.

REPORT OF CASE

A man, aged 41, had been instilling mild silver protein into both eyes daily for three years. It was first prescribed for a conjunctivitis, but as he found that it passed through the tear duct into his nose and thought that it made his attacks of coryza less frequent, he had continued to use it after the conjunctivitis subsided. I saw him first on Dec. 20, 1928, and at that time neither irritation nor symptoms due to the argyrosis was noted, but the color of the bulbar conjunctiva gave his eyes an unpleasant appearance. Examination showed a marked brownish gray pigmentation of the plical bulbar and palpebral conjunctiva of both eyes. The staining was more intense in the lower portion, as is usual. The two eyes were about equally affected.

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* Read before the Section on Ophthalmology at the Eightieth Annual Session of the American Medical Association, Portland, Ore., July 12, 1929.

[1] Fuchs, A.: Atlas of Histopathology of the Eye, part 2.

[2] Gettler, A. O.; Rhoads, C. P., and Weiss, S.: Am. J. Path. 3: 631 (Nov.) 1927.

[3] Stillians, A. W., and Lawless, T. K.: Intracutaneous Method of Treating Argyria, Arch. Dermat. & Syph. 17: 153 (Feb.) 1928; Argyria, J. A. M. A. 92: 20 (Jan. 5) 1929.

[4] Terlinck: Ophthalmic Year Book, 1909.

It was decided to try the solution on only one eye and to compare it with the other eye for results. Instillation of the mixture did not cause any irritation or loss of pigment, therefore, as a trial, on Jan. 17, 1929, 4 minims (0.24 cc.) of a mixture of equal parts of a 12 per cent sodium thiosulphate and a 2 per cent potassium ferricyanide solution was injected just under the conjunctiva of the lower fornix, for there a slough would have done little harm. An immediate lightening of pigment around the needle point was noted. At the time of the injection there was considerable stinging pain, which lasted from one-half to one hour. The next day there was very little reaction, and after three or four days one could note the markedly lessened pigment in the area injected. A stainless steel needle was used but the solution corroded it, so that it left a mark as a tattoo needle would have done. A platinum needle as advised by Stillians and Lawless was used for further injections. Anesthesia with 5 per cent cocaine was used, and when the plica was infiltrated, cocaine powder was dusted over the point of injection. Epinephrine was instilled before injection to slow down absorption.

January 18, several injections of from 2 to 3 minims (0.12 to 0.18 cc.) each were made in the lower fornix and plical regions, the same solution being used. There was marked clearing at the points injected and very little reaction. As the solution seemed so little irritating, I tried a subconjunctival injection of 10 minims (0.6 cc.) of a solution of two parts of 2 per cent potassium ferricyanide and one part of 12 per cent sodium thiosulphate in the lower bulbar conjunctiva, January 24. The solution was injected just beneath the conjunctiva and the point of the needle was moved around so that the fresh solution from the syringe would come into contact with the entire area. This was found to be quite important, as the solution has little effect on the granules if it has diffused through much tissue to reach them. The solution must also be used as soon as the two are mixed, for the mixture is unstable. The pain at the time of this injection was rather severe, but the use of infiltration anesthesia was found by Stillians and Lawless to lessen the effect of the injections. There was less reaction than one would get from a subconjunctival injection of the same amount of 5 per cent saline solution. By the next day the eye had become almost quiet and there had not been any pain one hour after injection.

February 6, the plica was injected with 10 minims of the two to one mixture. The reaction was again very slight. This injection so cleared the visible part of the conjunctiva that it was deemed inadvisable, because of the pain, to try to clear the palpebral conjunctiva. The cosmetic effect obtained was sufficient. I am not sure that the tarsal conjunctiva could be injected because of its close adherence to the tarsus.

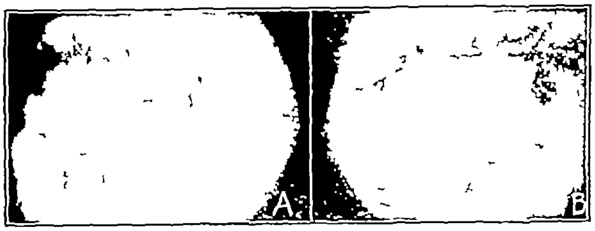

Fig. 2.—*A*, untreated eye; *B*, left eye after treatment.

February 11, five days after the last injection photographs reproduced in the accompanying illustrations were made. One can see that the reaction has completely disappeared from the plical injection in figure 2*B* as there is no hyperemia. There are still some traces of pigment in the plica but not enough to be conspicuous.

COMMENT

Figures 1, 2 and 3*A* show the untreated right eye, and *B* the treated left eye in approximately the same positions. The photographs were all made with one-half second exposure in the same light. The difference in figure 3 between *A* and *B* is most marked, as the pigment was most abundant in the lower portion of the conjunctiva.

The second eye is now being treated, and I am presenting this single case, for it proves beyond doubt that this method, while not perfect in its results, offers the first opportunity to remove silver deposits from the bulbar conjunctiva thoroughly enough to give a good cosmetic result. The thoroughness of removal depends on the willingness of the patient to stand the pain and discomfort of repeated injections. It has been suggested that morphine, or morphine and scopolamine, might be used in sensitive patients before injection.

The chemical action of these solutions is not clear, but M. E. H. Volwiler stated that it was likely that the silver was first converted into silver ferricyanide and then into sulphide by the thiosulphate. The excretion is then carried out just as thiosulphate removes mercury from the system. The exact action is not clear, but the clinical results are good.

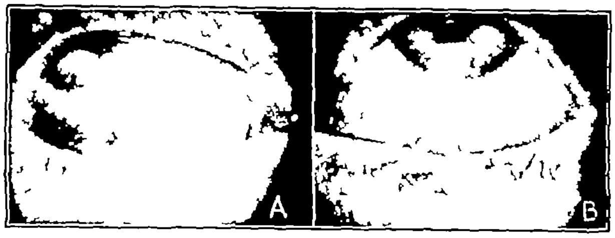

Fig. 3.—*A*, untreated eye; *B*, left eye after treatment.

TECHNIC

The most satisfactory technic may be given clearly in brief. Using sterile water and sterile bottles, one should first make a 12 per cent solution of sodium thiosulphate and a 2 per cent solution of potassium ferricyanide, *not ferrocyanide*. The solutions are not further sterilized. The conjunctiva is cocainized with a 5 per cent solution, and epinephrine is instilled. Two parts of ferricyanide solution are mixed with one part of thiosulphate solution, and from 10 to 15 minims (0.6 to 0.9 cc.) is drawn into a 2 cc. syringe with a number 26 platinum needle. The solution is injected as superficially as possible under the pigmented conjunctiva, the needle point being moved to as many different areas as possible while the fluid is being injected. After withdrawal of the needle, the bleb should be massaged thoroughly through closed lids. The injections are repeated wherever the pigment is most dense. In areas in which two injections overlapped, there was some further clearing on the second injection.

I wish to emphasize again the danger of argyrosis from the prolonged use of mild silver protein and the colloidal silver solutions.

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ABSTRACT OF DISCUSSION

Dr. W. D. Horner, San Francisco: The laity and, to a lesser extent, the corner druggist have three sterling remedies for the treatment of any eye disorder, namely, boric acid, mild silver protein and ointment of yellow mercuric oxide. Boric acid and yellow mercuric oxide are not harmful to the eye save that their use may preclude a proper diagnosis of the disorder, thereby wasting valuable time and possibly jeopardizing the eye. The whole series of silver protein preparations must, however, be used with some caution. Two cases of generalized argyrosis due to the prolonged use of silver protein as nasal drops were recently reported. The drops had been prescribed by the pediatrician for coryza but the mother continued to use them long after the acute condition was cured in the hope of warding off future colds Generalized argyrosis was the result

One of my colleagues in San Francisco recently told me of a case of permanent staining of the cornea which resulted from prolonged application of silver protein to an extensive corneal abrasion. Argyrosis of the conjunctiva is commonly encountered In our experience the majority of these cases have occurred in chronic tear sac infections in which the patient endeavored to clear up the purulent discharge and the accompanying conjunctivitis by long continued applications of silver protein

I think that general practitioners and pediatricians are much too careless in the frequency with which they prescribe silver compounds Known to the laity as perfectly harmless preparations, experience has shown that they are not harmless There are probably few medicine chests in this country which do not contain a bottle of mild silver protein, more or less ancient, from which copious and reckless applications are made to eyes, noses and throats as occasion arises

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EMPHYSEMA SIMULATING CARDIAC DECOMPENSATION *

WILLIAM B KOUNTZ, M D
H L ALEXANDER, M D
AND
DONALD DOWELL, M D
ST LOUIS

Most of the modern textbooks of medicine state that emphysema leads to heart failure This is presumed to be due to stretching and later, as the lungs distend, to rupture of the pulmonary capillaries in the alveolar walls It is thought that the reduction in the vascular bed thereby throws on the right side of the heart an extra load which leads to hypertrophy and dilatation and subsequent right-sided heart failure With this reasoning, the dyspnea, cyanosis and dependent edema so often seen with advanced emphysema are explained as the results of cardiac decompensation Hoover,[1] however, found little evidence of pure rightsided failure He believed that both the right and left ventricles are affected and attributed their failure to the effects of anoxemia on the heart muscle

Alexander, Luten and Kountz [2] reported fifty cases of long standing bronchial asthma, most of which were complicated by pronounced emphysema In this series, direct evidence of injury to the heart was singularly lacking

In order to determine whether the heart is injured as a result of emphysema, the study reported here was undertaken

Sixty-six patients with advanced emphysema were examined The underlying causes were varied and included bronchial asthma, chronic bronchitis, tracheal compression by tumor and thoracic enlargement with Paget's disease The routine examination of all patients included a history with particular emphasis on cardiac symptoms, physical examination, an electrocardiogram, a 6 foot roentgenogram of the chest, measurement of vital capacity, determinations of arterial and venous blood pressure and, in eighteen cases, an evaluation of the degree of oxygen unsaturation in arterial blood

Of the sixty-six patients, fifty-eight had cyanosis and dyspnea on exertion In eighteen, edema was also present and the clinical picture of cardiac decompensation seemed complete Twenty-one other patients gave a history of edema which was, however, not observed while they were in the clinic Thirty-nine cases (59 per cent), therefore, either showed a dependent edema or gave a history that it had been present All of them at one time or another had been treated for heart trouble

It was frequently noted that the dyspnea, cyanosis and edema became decidedly worse when there were acute exacerbations of the underlying pulmonary disorders This occurred, for instance, in bronchitis with continued cough or in bronchial asthma after a patient had had a series of asthmatic attacks As these symptoms improved, so also did the dyspnea, cyanosis and edema The edema was always dependent and rarely extended above the knee.

The degree of emphysema, as measured by the vital capacity of the lungs, was considerable in all cases, and in some was less than 50 per cent of the patient's estimated normal Despite this, there were surprisingly few laboratory observations that pointed to heart trouble These were as follows

In five instances the electrocardiogram showed right ventricular predominance In two of these cases which came to autopsy, no cardiac enlargement was found The heart was displaced to the right in one by adhesions and in the other by a chest deformity There was a generalized enlargement of the cardiac shadow in the 6 foot roentgenograms of eight patients One of these came to autopsy Both the right and left ventricles were found hypertrophied and dilated In this case there was some arteriosclerosis which included the coronary arteries The heart muscle showed some fibrosis but no other microscopic pathologic condition Two other patients with enlarged hearts had syphilis, but without aortic insufficiency Two had definite arterial hypertension It is noteworthy that in the entire series only these two patients had blood pressures above the normal range

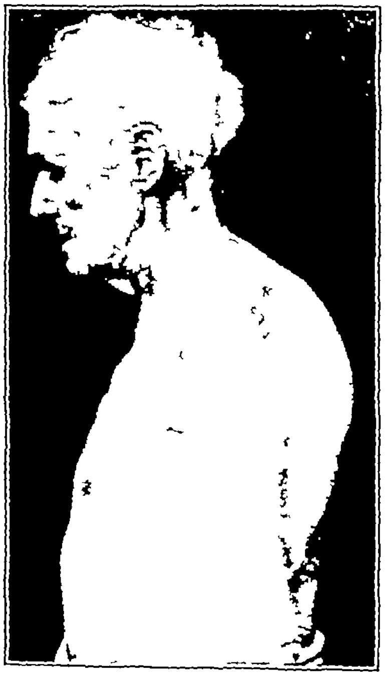

Fig 1—Lateral view of chest, showing marked deformity from emphysema

In most cases there was an elevation of peripheral venous pressure which ordinarily seemed roughly proportionate to the degree of bronchial obstruction It would rise markedly during asthmatic attacks or during paroxysms of cough On the other hand, in Paget's disease and in spinal arthritis, in which the primary difficulty was in the bones, normal venous pressures were obtained even though thoracic expansion

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* From the Department of Internal Medicine Washington University Medical School City Hospital and Barnes Hospital

[1] Hoover C F in Blumer's System of Medicine 2:172 1928

[2] Alexander H L, Luten Drew and Kountz W B Effects on Heart of Long Standing Bronchial Asthma J A M A. 88 882 (March 19) 1927

was extreme under these conditions Here the lungs "followed" the thoracic deformity and were not the cause of it This important relationship is discussed later

The degree of oxygen unsaturation in the arterial blood was determined in eighteen cases It varied from normal to 40 per cent unsaturation, depending largely on the clinical symptoms at the time the blood was taken During an exacerbation of bronchitis or asthmatic paroxysms the oxygen content of the arterial blood would drop, only to rise again as the patient improved In extreme emphysema it remained consistently low

Nine of the sixty-six cases came to autopsy and a detailed study of the heart in each instance was made Although the total heart weights varied the normal ratio of right ventricular weight to left ventricular weight was maintained in all instances In one case in which it approached the upper limit of normal, there was sclerosis of the pulmonary artery In another, there was hypertrophy and dilatation of both right and left ventricles, with fibrosis of the heart muscle as has been mentioned In the remaining eight cases there was no evidence either of right or of left ventricular dilatation or hypertrophy or of excessive fibrosis The sudan III stain did not reveal any fat

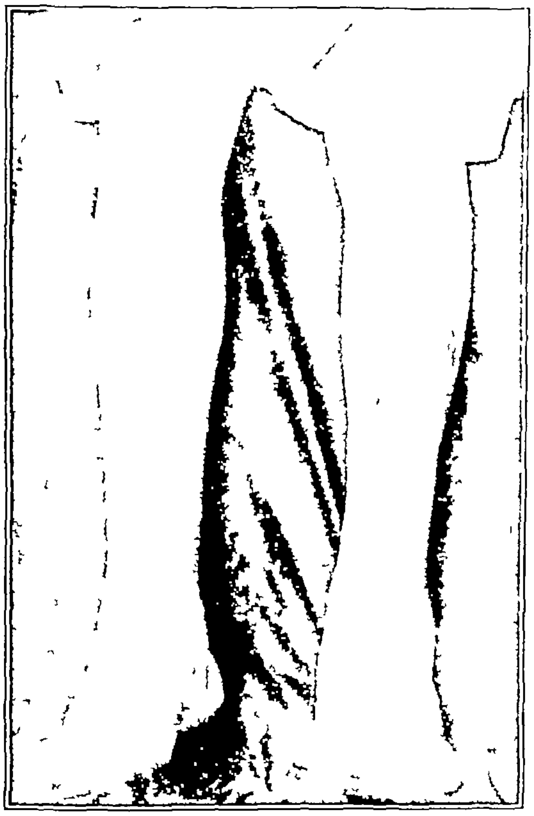
Fig 2 —Same patient showing dependent edema

In all cases the lungs showed emphysema, more marked at the apexes and lung margins where huge emphysematous blebs often occurred beneath the pleural surface Microscopically, the lesions were characteristic

Since in eight cases that came to autopsy there was no evidence of cardiac impairment, it was necessary to explain the dyspnea cyanosis and edema in some other way The dyspnea and cyanosis could be attributed to the lowered vital capacity, which resulted in a reduced oxygen content of the arterial blood The edema was ascribed to two factors increased peripheral venous pressure and anoxemia

Although high venous pressures occur in heart failure, some other mechanism must be assumed to account for its occurrence in these fatal cases of emphysema The problem was studied by inducing emphysema in dogs by means of an adjustable ball valve in the trachea[3] In this way, an extensive emphysema may be produced within a few weeks In a series of sixteen dogs so treated, simultaneous determinations of intrapleural and peripheral venous pressures were made at intervals for several weeks It was found that, as emphysema developed, the intrapleural pressure rose This was followed by a rise in peripheral venous pressure The curves of intrapleural and venous pressure roughly paralleled each other (fig 3)

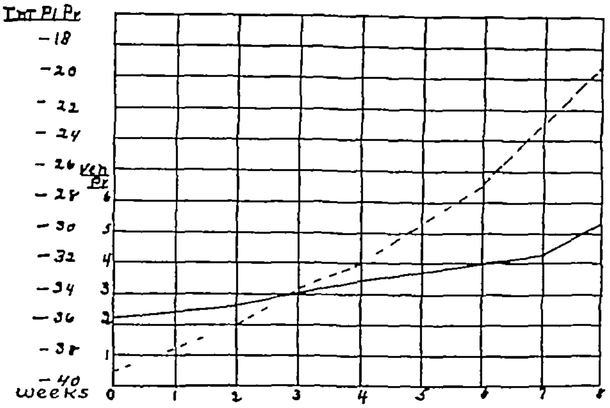

Fig 3 —Curves showing the rise of the venous and interpleural pressures in a dog with a ball valve in the trachea The pressures were taken simultaneously twice a week Measurements are in cubic centimeters of water Solid line venous pressure broken line, interpleural pressure

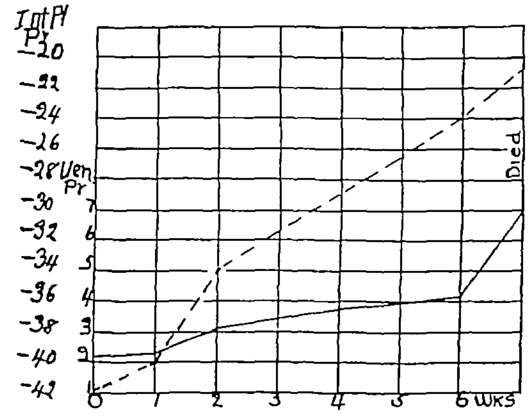

Fig 4 —Curve of the venous and the interpleural pressures of a dog with a ball valve in the trachea The pressures were taken twice a week and were measured in cubic centimeters of water A slow but gradual rise of the venous pressure occurred until the end of the sixth week, when it increased out of proportion to the interpleural pressure. The animal at the time developed a marked air hunger The oxygen content of the arterial blood was found to be reduced 40 per cent below normal Solid line venous pressure broken line interpleural pressure

As the flow of venous blood to the right side of the heart depends, in part, on the negative pressure within the thorax, any mechanism that lessens this negative pressure tends to block the venous return The obstructed venous flow is thereby reflected in an increased peripheral venous pressure Carrying this further, it is conceivable that the right side of the heart, instead of being overfilled in emphysema, actually receives less blood than normally and thus does not hypertrophy from overwork This suggestion does not take into consideration the possibility of obstruction in the pulmonary system, but at present there is very little accurate knowledge concerning the flow of blood through the lungs in emphysema It is interesting, however, that Blumgart and Weiss[4] have shown that there is obstruction only in the more advanced cases

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[3] Harris W H and Chillingworth F P J Exper Med 30 75 (July) 1919

It was noted during the course of these experiments that, when an animal developed so much emphysema that he became dyspneic on very mild exertion, the venous pressure would then rise out of all proportion to the intrapleural pressure (fig 4) Under these circumstances, the degree of oxygen saturation of the arterial blood was always found to be reduced below 70 per cent of the normal

Statistics on Nine Patients with Extreme Emphysema Who Died

Case	Clinical Diagnosis	Age	Electrocardiogram	Surface Area of Heart	Vital Capacity	Percentage of the Estimated Normal Vital Capacity	Arterial Blood Pressure	Blood Pressure (Venous) in Cc of Water	Edema	Oxygen Content of Arterial Blood per Cent by Volume	Per Cent of Oxygen Saturation of Arterial Blood	Carbon Dioxide Content of Arterial Blood per Cent by Volume
1	Bronchitis	64	RVP	Normal	2,200	48	130/80	9 12*	3+	14 11*	77 64*	47 50*
2	Asthma	60	Normal	Normal	1,800	48	105/72	9.5	2+	14	82	56
3	Bronchitis	65	RVP	Normal	1,500	48.5	148/95	10.5	2+			
4	Asthma	52	Normal	Normal	1,400	47	117/85	10.75	3+	11	70	52
5	Bronchitis	58	Normal	Normal	2,400	62	109/80	7.5	4+			
6	Bronchitis	64	LVP	Normal	600	66	160/95	9.5	3+	14	70	54
7	Asthma	51	Normal	Normal	1,560	47	118/80	10	3+			
8	Asthma	60	Normal	Normal	1,400	43.8	130/85	13.5	3+			
9	Asthma	48	LVP	Large	2,000	45	120/[illegible]	14	3+			

* During exacerbation of symptoms

Although no gross edema occurred in the experimental animals, it is noteworthy that both Krogh[5] and Landis[6] found an increase of blood capillary pressure in anoxemia Landis believed this condition could lead to a passage of fluid through the capillaries into the tissues

COMMENT

Several cases of advanced emphysema were found in which peripheral signs of cardiac decompensation appeared without demonstrable heart lesions This by no means implies that emphysema and heart failure do not coexist for it is common experience that patients with arteriosclerotic heart disease are apt to have emphysema also In these cases the cardiac symptoms are the prominent ones and the emphysema is usually not extreme. The cases that comprise the series reported here are selective in that this is a study of advanced emphysema Consequently, the most pronounced cases were chosen and these had a definite etiology ascribable either to a bronchial lesion or to one of the chest wall As a consequence, the moderate emphysema coincident with senile heart conditions did not naturally fall into this group There is no reason to believe that the heart failure in such cases is attributable to the associated emphysema

Moreover, no implication is made here that marked emphysema prevents heart failure One case appears in this series Hoover noted several Harkavy[7] reported one and others have been described[8] It is noteworthy, however, that in these cases of failure both the right and left ventricles were hypertrophied This implies some mechanism other than reduction of the pulmonary vascular bed The suggestion made by Hoover of a prolonged anoxemia of the heart muscle seems plausible Of particular interest is the rarity of authentic reports in which only right-sided lesions of the heart occurred

CONCLUSION

It is believed that, despite peripheral signs which simulate cardiac decompensation advanced emphysema does not necessarily affect the heart When there are cardiac lesions which might be attributable to the effects of emphysema it appears that they are due to a bilateral change rather than to involvement of the right chamber alone

600 South Kingshighway

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[4] Weiss Soma and Blumgart H L J Clin Investigation 4 555 (Oct) 1927

[5] Krogh August Anatomy and Physiology of the Capillaries ed. 1 New Haven Conn Yale University Press 1924

[6] Landis E M Am. J Physiol 81 124 (June) 1927 83 528 (Jan) 1928

[7] Harkavy J Proceedings Soc. Asthma and Allied Conditions to be published.

[8] Huber H L and Koessler K K Pathology of Asthma Arch Int Med 30 689 (Dec) 1922 Kountz W B and Alexander H L Death from Bronchial Asthma Arch Path. 5 1003 (June) 1928

CARCINOMA OF THE COLON *

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RICHMOND, VA

Cancer in readily observable regions, as the mouth, arises from a preexisting benign lesion, a papilloma, a small ulcer or a point of constant irritation It seems probable that this holds good in the more inaccessible regions of the body, where early observation is difficult or impossible In a patient of mine, Mrs C C S, with advanced cancer of the transverse colon, there were several small adenomas near the cancer (figs 1 and 2) The histologic structure of one of these adenomas showed that, while the growth was benign, there was a tendency to hyperplasia of the epithelial cells (fig 3) It is a logical conclusion that the cancerous lesion of the colon originated in an adenoma like this

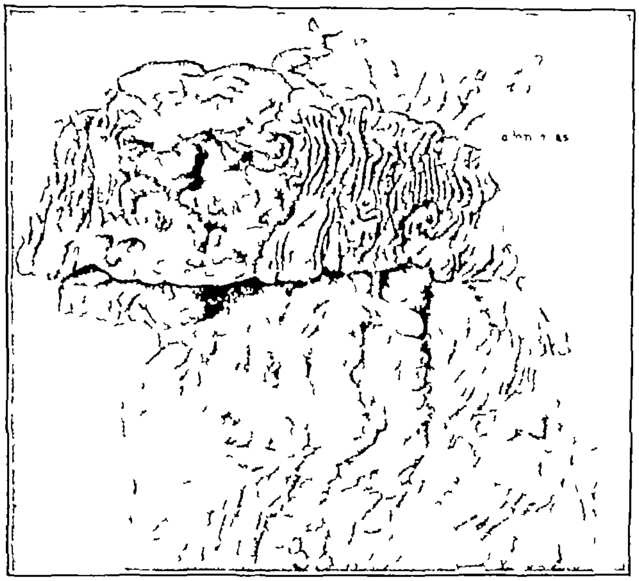

Fig 1 —Advanced carcinoma of left side of right colon in Mrs. C C S Several benign adenomas were in the mucosa adjacent to the growth The structure of one of them is shown in figure 3 It seems probable that the carcinoma developed from an adenoma like one of these.

SYMPTOMS AND SIGNS

Often the first symptom of carcinoma of the colon is obstruction This is usual when there is a scirrhous cancerous stricture A disturbance of the bowel movements from what is normal for the patient, constipation sometimes followed by diarrhea, and the passage of blood and mucus, may be early symptoms The passage of blood, if fresh and clotted, particularly if there are no hemorrhoids, is always of grave significance Tarry stools usually come from the upper intestinal tract or from the stomach Pain occurs chiefly when there is necrosis of the cancer and a surrounding inflammatory reaction involving the peritoneum A peculiar feature of carcinoma of the right side of the colon is anemia, which may resemble closely pernicious anemia This is due not to any intrinsic property of the growth itself but to the fact that absorption of fluids in the right colon is much more rapid than in the left colon, and, consequently, the effect of the greater absorption of hemolytic toxic products from the necrotic cancer of the right colon is more marked The tumor is often palpable

Roentgenologic examination is an important factor in the diagnosis The barium should be given as an enema If the growth is in the rectum, digital and proctoscopic examination is all that is necessary, but, if beyond the reach of the finger or proctoscope, a careful roentgenologic examination with a barium enema is of great value A tumor in the left half of the transverse colon may prolapse to the region of the sigmoid when it is almost impossible to tell by palpation whether it arises from the sigmoid or from the transverse colon A roentgenologic examination will clear this up and enable the surgeon so to place the incision as to secure the best accessibility

PATHOLOGIC CONSIDERATIONS

The histologic examination of cancer of the colon greatly aids the making of a correct prognosis for cure after a surgical operation The classification of cancer by A C Broders into grades of malignancy assists both the pathologist and the surgeon But histologically very malignant cancers sometimes meet a marked resistance and an early operation can cure, while the mild types that have become extensive would be refractive to any treatment

Cancer of the colon may be a slowly growing scirrhous type, it may advance fungus-like into the lumen of the colon, or it may be a large infiltrating tumor whose center breaks down and forms a cavity lined with necrotic foul material The latter type is accompanied by infection and frequently becomes adherent to the surrounding structures Adhesions do not necessarily mean an extension of the cancer, they often occur from accompanying saprophytic and pyogenic bacteria. In such cases, however, an infection may result from separating the adhesions and ligation of the mesentery whose lymphatics are filled with bacteria from the necrotic area, and frequently a fatal peritonitis begins A resection with immediate anastomosis is often fatal in these cases Even if the peritonitis is overcome, the sutures usually break down and a fecal fistula results Some types of cancer grow in an encephaloid or fungous manner, projecting into

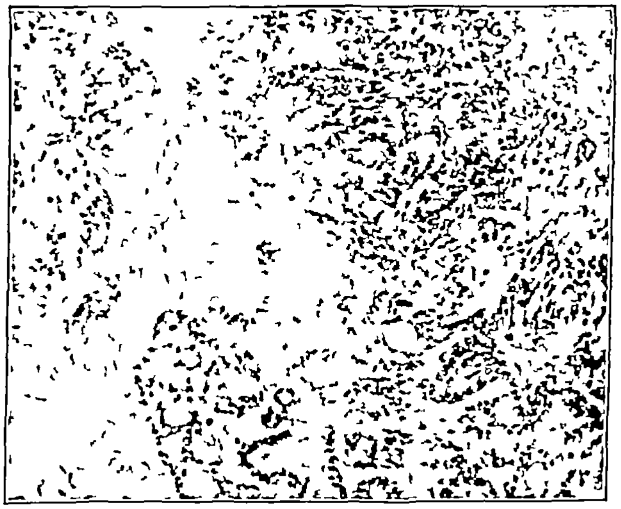

Fig 2 —Adenocarcinoma of Mrs C. C. S. (specimen shown in figure 1) about grade 2½ reduced from a photomicrograph with a magnification of 140 diameters.

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* From the Surgical Department of St. Elizabeth s Hospital

* Read before the Section on Surgery General and Abdominal at the Eightieth Annual Session of the American Medical Association Portland Ore., July 11, 1929

the lumen of the bowel, and bleed freely When the histologic examination shows a colloid structure the cancer may vary grossly from a firm hard mass to a soft tumor Only a portion of the tumor may be colloid As Broders has pointed out, the colloid itself is of no especial significance in indicating the grade of malignancy If the colloid is derived from cells that are well differentiated and columnar, the cancer may be considered mild, but if the colloid comes from poorly differentiated "signet ring" cells, the prognosis is bad as it indicates a high grade of malignancy

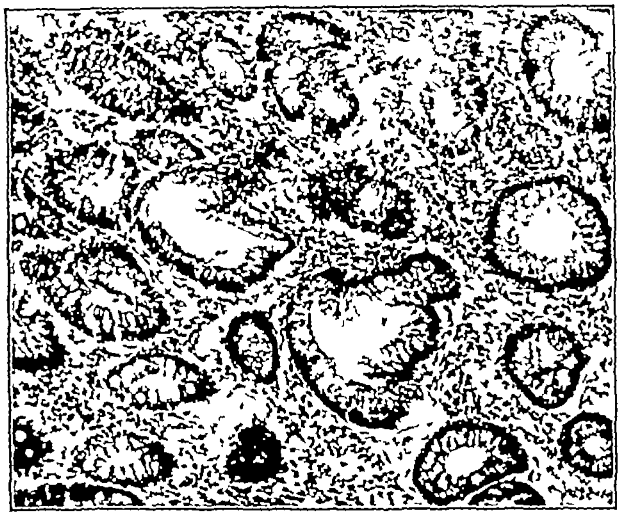

Fig 3—Appearance of one of the adenomas shown in figure 1 The tendency toward hyperplasia of the epithelium is apparent even though the growth is benign There is also a marked leukocytic infiltration. It seems highly probable that the carcinoma shown in figure 1 arose from one of these adenomas Reduced from a photomicrograph with a magnification of 140 diameters

Cancer of the colon may kill by obstruction, by hemorrhage, by toxemia or sepsis, particularly when on the right side, by metastases through the lymphatics or through the portal system, or by a combination of these factors The lymphatics are not so abundant or active as in the stomach, and metastasis is usually late Even when the growth is extensive, radical operations may sometimes be attempted which would not be justified in a similarly extensive cancer of the stomach

TREATMENT

Surgical excision probably gives the only chance of cure for cancer of the colon, though competent radiologic therapy may greatly aid

The type of operations best suited for malignancy of the colon depends on several factors, the extent and nature of the growth, the general condition of the patient, and the presence or absence of obstruction In acute obstruction when the cancer is small and its location is not definitely known, it is best to make an incision close to the midline As Sistrunk[1] has pointed out from his experience in ulcerative colitis, extensive manipulation is unwise, and yet a gentle examination with the hand to determine the location of the neoplasm and whether there are metastases can be done without too great risk This knowledge is important If it is found that the tumor is inoperable, a different type of enterostomy is indicated from that which is done in an operable growth If it is inoperable, a large open colostomy is made preferably near the growth in order to prevent a blind pocket being formed between the cancer and the enterostomy If it is operable but obstructive, frequently a Witzel type of cecostomy with a rubber tube is all that is necessary This gives drainage from the cecum in which fecal material is normally liquid, and also permits flushing out of the colon either throughout its whole length, if the growth becomes permeable, or at least through the portion proximal to the tumor The indications for relief of obstruction and for cleansing the bowel are thus fairly well met This also minimizes the danger of soiling in a subsequent operation for resection

PRELIMINARY PREPARATION

If there is an acute obstruction, nothing more should be done than an enterostomy If the cancer is operable it is excised after two or three weeks of general preparation During this interval the diet is important The patient should receive an abundance of fluids, and enemas of salt solution several times a day, with irrigations through the enterostomy tube If the anemia is marked a transfusion of blood may be given The intravenous injection of 5 per cent dextrose in Ringer's solution, from 1,000 to 2,000 cc a day, is often helpful[2] In a cecostomy, dextrose and salt solution can be instilled through the cecostomy tube, which is clamped for a while to admit of absorption If this procedure, however, makes the patient very uncomfortable it should not be continued Practically all animal proteins should be prohibited The diet should consist largely of fruit

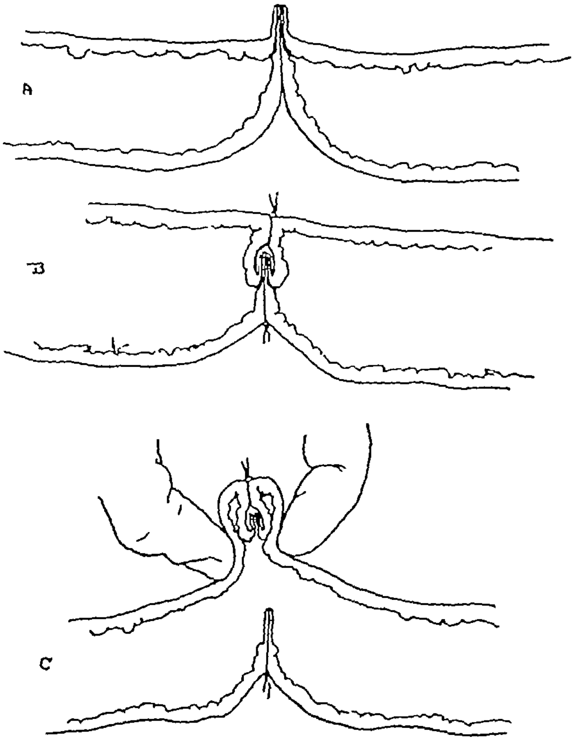

Fig 4—Diagrammatic sketch of procedure referred to in the text in which both loops of the bowel are clamped by a *single stout* Payr clamp The diseased segment of the bowel is removed Sutures are applied and the diaphragm is broken up by invagination of the thumb and finger The chief theoretical advantage is that the mesenteric border of the segment remains agglutinated and only one stout clamp is used. Practically it was found that the nutrition of the bowel ends could not be satisfactorily adjusted, and consequently the method was dangerous.

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[1] Sistrunk, W E Factors of Safety in Operations for Carcinoma of the Colon J A M A **91** *1800 1801* (Dec. 8) 1928 The Mikulicz Operation for Resection of the Colon Ann Surg **88** 597-606 (Sept.) 1928

[2] Horsley J S Operative Surgery ed. 3 St. Louis C. V Mosby Company 1928 pp 86-88 The Influence of Physiologic Research on Modern Surgery Long Island M. J, March 1929 pp 139 145

juices, carbohydrates, sugar, candy and rice, which not only leave little residue but prevent the bacterial growth that accompanies the ingestion of protein. Water is abundantly given. This is not a well balanced diet and cannot be continued indefinitely, but for a few weeks the patient will improve on it. This diet is also indicated as a preliminary preparation for excision of nonobstructive colonic cancer.

Rankin and Bargen,[3] in a study of sixty cases in which vaccine therapy was employed in addition to other preparation, found a mortality rate from postoperative peritonitis of only 5 per cent, against a 23 per cent mortality rate in a similar group of cases without the preliminary vaccination. The vaccine was prepared from colon bacilli and streptococci of cases of fatal peritonitis and was given intraperitoneally.

SOME TECHNICAL POINTS

If the patient is fat and the mesentery is strutted out and short, or if the tumor is large, necrotic and adherent, primary anastomosis after resection should not be considered. Usually it is possible in some way to make a union of the stumps of the bowel unless the resection is in the lower sigmoid, but whether this should be done as a primary operation or in stages after the manner of the Mikulicz procedure depends on the conditions present. When the bowel and mesentery are very fat, sutures, no matter how carefully inserted, will break down and the most favorable outcome would be a fecal fistula. Often a fatal peritonitis develops. If the patient is not fat and the growth is small with no obstruction, a primary anastomosis may be done, especially if there has been some preliminary preparation. In doubtful cases, however, it would be best to do the operation in multiple stages.

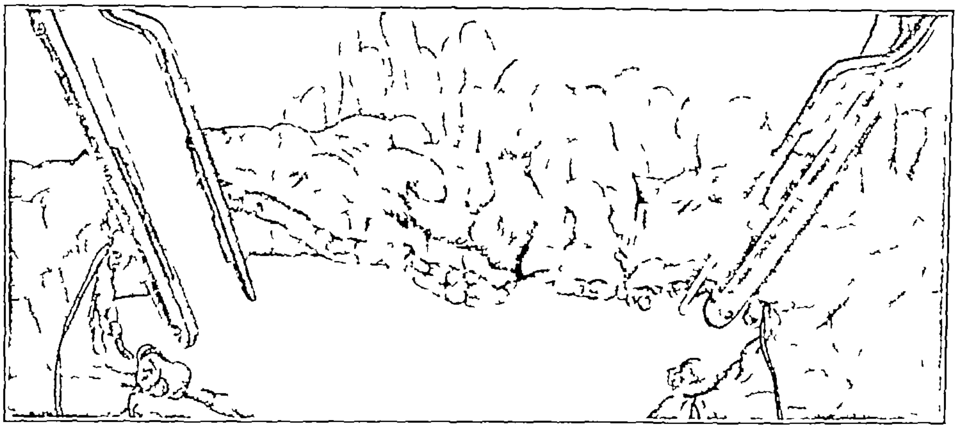

Fig. 5.—First stage in resection with primary anastomosis of the colon. The mesentery has been divided and tied and the bowel is clamped and one end has been divided. The clamps are applied so that the mesenteric borders will not be in apposition.

If a primary anastomosis is indicated, the procedure should preferably be carried out according to the so-called aseptic technic. This method was first brought into prominence by Kerr[4] and Parker. The bowel at each end of the loop to be resected is doubly clamped and divided with the cautery, the stumps being closed with a basting stitch and the ends then approximated and sutured together. When the suturing has been completed, the basting stitch of each stump is withdrawn.

Before the bowel is divided in any technic of resection, the mesentery is severed and ligated and the loop to be removed is thoroughly mobilized. An important consideration, particularly in resection of the colon, is the blood supply. On account of the active physiologic function of the upper small intestine, its blood supply is much more abundant than that of the colon. In the colon, and especially in the transverse and left colon where the blood supply is scanty, suturing will often break down unless it is seen that there is satisfactory vascular nourishment of the tissues. Not infrequently ligation of the vessels of the mesentery that appears not to interfere with the nutrition of the stump of the intestine will be found to interfere very materially, and one side or both sides of the anastomosis may become necrotic. To avoid this, cutting the mesentery parallel with the bowel at its attachment until free bleeding occurs insures a good blood supply to the sutured tissues.

Though the principle of "aseptic" intestinal anastomosis is commendable in operations on the colon, there are several objections to the technic Kerr employs. One is that the insertion of the basting stitch may in spite of care penetrate the lumen and cause soiling. Then, too, when the stumps have been folded in and sutured the basting stitch must be withdrawn. Occasionally the basting stitch hangs and is difficult to remove. If it has penetrated the lumen at any point, naturally its withdrawal may spread infection. Another objection to this technic is that so much infolding of the ends of the colon by the basting stitch and still more by the permanent sutures tends to occlude the bowel. This encroachment on the lumen may not be as serious in surgery of the colon as in operations on the small intestine, but it is not without a distinct disadvantage in this region and while it will not cause permanent obstruction in the colon of a dog, in man whose intestinal muscular development is much weaker than in a dog, it may be fatal.

Rankin[5] has devised an ingenious three-bladed clamp especially adapted for colonic resection. The bowel is brought up and clamped at each end of the diseased loop after the manner of the old Roosevelt clamp for gastro-enterostomy. The loop is cut off with the cautery, and sutures are placed around both sides of the clamp, which is removed while the anterior basting suture is held taut. When the bowel is not fat, this clamp doubtless is advantageous. As Rankin has pointed out, too, as a step in the Mikulicz operation this clamp may be helpful. Sometimes, however, it is difficult to place sutures accurately on the under side of the bowel when this clamp is in position. Another and a more serious objection is that the colon cannot be easily adjusted in this clamp so that the best nutrition at the stumps will be secured This is more readily accomplished when separate clamps are placed on the ends

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[3] Rankin, F. W., and Bargen, J. A.: Intraperitoneal Vaccination in Cases of Carcinoma of the Colon by Mixed Vaccine of Colon Bacilli and Streptococci, read before the Southern Surgical Association, White Sulphur Springs, Dec. 11, 1928.

[4] Kerr, H. H.: Development of Intestinal Surgery, J. A. M. A. **81**: 641-647 (Aug. 25) 1923.

[5] Rankin, F. W.: Surgery of the Colon, New York, D. Appleton & Co., 1926.

In an effort to make the operation simpler I have clamped together both limbs of the loop of bowel with one stout Payr clamp This would also appear to have the advantage of leaving the mesenteric borders of the stumps still adherent by the pressure of the clamp (fig 4) The procedure was not successful however, because it was found that in slipping backward and forward an adjustment of the bowel was apt to be made that impaired the nutrition of the stumps

PRIMARY ANASTOMOSIS

In two cases I have done an operation which is a combination of some of the features of the principle of the aseptic technic of Kerr and the method of Rankin, and the results so far as the anastomosis was concerned have been satisfactory In the first case the patient died about four weeks after the operation from an abscess around the liver When the postmortem specimen was examined, the anastomosis was in excellent condition and there was no stricture In another case there was a fecal fistula but much of the left colon and sigmoid had been removed, and though the patient was fat an immediate end-to-end union seemed the only way to avoid a permanent artificial anus

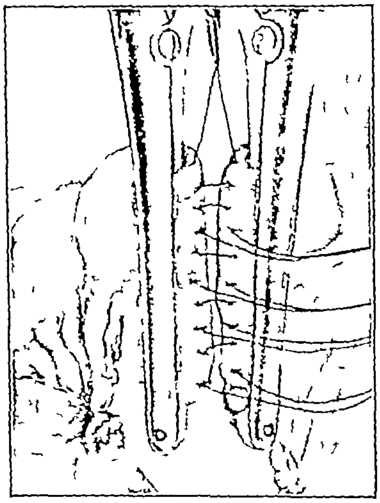
Fig 6—The stumps of the bowel are brought together A series of interrupted mattress sutures has been applied. They are all applied before any one is tied.

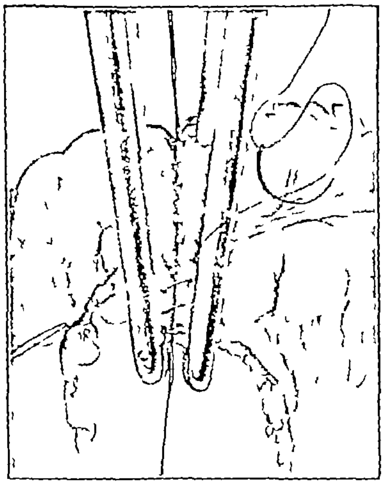
Fig 7—The mattress sutures are tied and a row of continuous sutures is applied

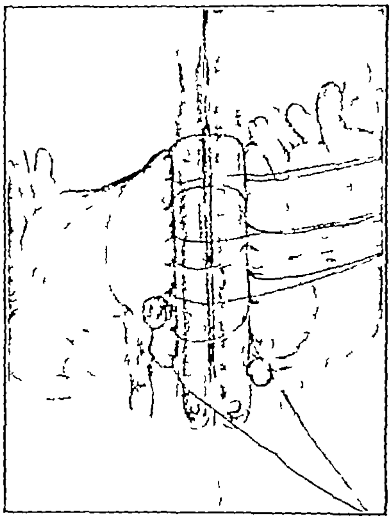
Fig 8—Interrupted mattress sutures have been applied anteriorly

As the first step in this operation, the loop of bowel to be resected is thoroughly mobilized by dividing and ligating its mesentery Payr clamps are applied in such a manner as to preserve the nutrition of the ends of the bowel Preferably, one clamp is placed at about right angles to the mesentery and the other parallel with the mesentery (fig 5) This obviates the necessity of having the raw surfaces on the mesenteric border of the two stumps in contact The bowel is resected with the electric cautery, and the stumps in the clamps are thoroughly cooked The two clamps are brought together and a series of mattress sutures of linen silk or chromic or tanned catgut is placed on the under surface before any of the sutures are tied (fig 6) After the sutures have been placed, the tension is relieved by approximating and slightly everting the clamps and then the sutures are tied A row of continuous sutures, preferably of silk or of linen, is taken closer to the clamp (fig 7) A series of interrupted mattress sutures is applied over the anterior surfaces of the clamps, care being taken to cover the bowel at the two extremities of the suture line These are held loosely while the clamps are gently withdrawn (fig 8)

Rankin has observed as a result of experimental work that the Payr clamp will usually leave the bowel walls stuck together if the clamp is carefully removed The application of petrolatum on the clamps before they are applied might aid in this After the Payr clamps are removed the mattress sutures are tied The mattress sutures should not be held too taut while the clamps are being opened Not infrequently if a basting stitch is applied the opening of the clamp sufficient for its removal will pull open the bowel by making traction on the basting stitch This is to some extent avoided when interrupted mattress sutures are used (fig 9)

After these mattress sutures are tied, a row of continuous sutures, preferably of tanned or chromic catgut, is placed (fig 10) There are, then, two rows of sutures, posteriorly an internal continuous suture, and anteriorly internal interrupted mattress sutures Any weak points are reinforced by additional sutures, and the diaphragm is broken by invaginating the bowel above and below the anastomosis with the thumb and finger

When the cecum and right colon are excised, the sutures should be taken at a higher level on the ileum than on the colon In this way an invagination occurs and an ileocecal valve which to some extent prevents reflux from the colon into the ileum, is reconstructed

MULTIPLE-STAGE OPERATION

When a primary anastomosis is not indicated, some kind of a multiple-stage Mikulicz operation should be done The classic Mikulicz operation is in four stages The affected loop of bowel is first brought onto the abdominal wall A few days later the loop of bowel is excised Still later a clamp is applied to the spur between the two stumps, and in the fourth procedure the external fistulous opening is closed There are modifications to suit the individual case

One of the dangers sought to be avoided in this procedure is peritonitis This operation is particularly applicable in large adherent neoplasms or in patients who are quite fat The stages, however, may often be advantageously modified It seems ideal to remove the neoplasm as soon as possible, not only because it is always a source of contamination by cancer cells but because toxic material from it, particularly when the blood supply has been interfered with, is not good for the patient In the first stage it is advisable to mobilize the bowel as though a primary resection were to be done The adhesions are severed The mesentery is divided and ligated, and the loop to be resected is brought easily into the wound Instead of following the formal Mikulicz procedure of leaving the loop for a few days until adhesions have occurred, the adjacent limbs of the loop are loosely sutured together in such a manner that the mesentery is not included in the spur, and then the affected loop is clamped with two Payr clamps or with the Rankin clamp and severed with the electric cautery The raw surfaces where there were adhesions, and the stumps of the mesentery, are drained with cigaret drains and a tube. This is important because peritonitis may follow from the separation of adhesions or division of the mesentery in these cases The clamps are left on the ends of the bowel and a Witzel cecostomy with a tube is done The clamps should not be removed for at least five days, by which time the drainage has been taken out and the cavity left by the drainage has contracted Three or four days later a clamp is applied to the spur (fig 11) After this, depending on the reaction of the tissues, an interval of from one to several weeks is permitted to pass and the external opening is sutured [6]

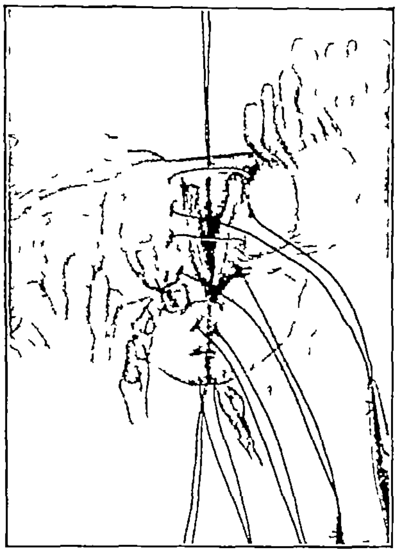

Fig 9 —The clamps have been withdrawn and the interrupted mattress sutures are being closed down and tied.

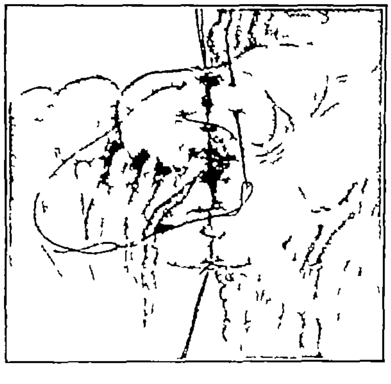

Fig 10 —A row of continuous sutures is applied burying the interrupted mattress sutures The diaphragm is then invaginated with the thumb and finger

The important advantage of this procedure is that excision of the cancer and drainage of the potentially infected tissues are done at the first stage, the other steps being minor procedures

617 West Grace Street

ABSTRACT OF DISCUSSION

Dr. R. C Coffey, Portland, Ore "Of all sad words of tongue or pen, the saddest are these, 'It might have been'" There is nothing that tears the heartstrings of the surgeon more in the case of simple annular carcinoma of the colon than to realize that he has sacrificed his patient's life to idealism. Idealism in intestinal surgery is the most fatal of all sentiments Less than three months ago I had a beautiful case of annular carcinoma of the upper sigmoid I knew there was danger and called attention to the fact in my clinic at the time that it was a dangerous thing to do, but it seemed such a perfectly simple case, I decided on ideal resection Two or three days later an intestinal loop apparently became caught in the neighborhood of the anastomosis and the patient died from a high intestinal obstruction I have many times successfully performed radical resection in such cases, but the mortality is too great when compared with the Mikulicz operation. It is probable that there has never been so important a principle introduced into intestinal surgery as the Mikulicz principle. It does not really matter, if the patient's entire future is under consideration, whether he is in the hospital one week, three weeks or two months Safety above all things should be the first consideration True, I still occasionally do the ideal resection, but I confess I have great difficulty in determining that fine hair line of wise decision Dr Balfour's tube technic is adapted to very low sigmoid cancer In this case, the pathol

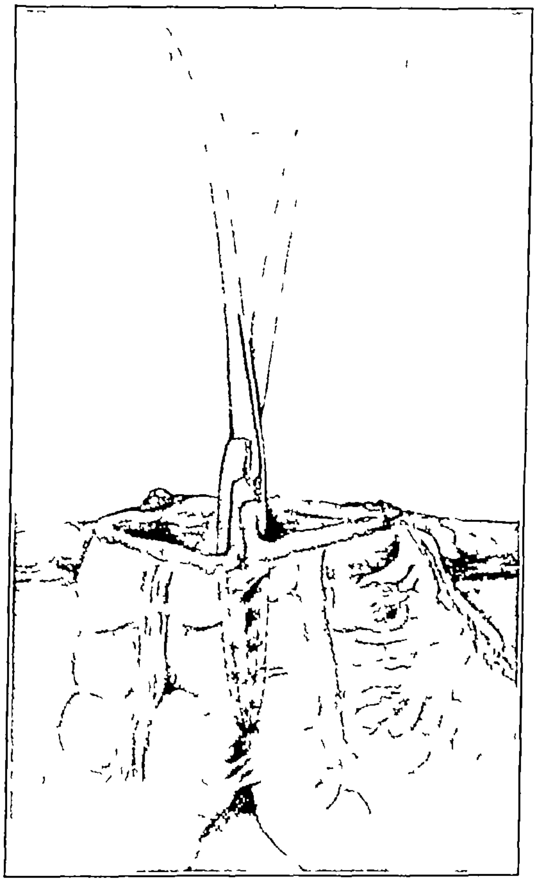

Fig 11 —The bowel has been brought up and resected and the clamps have been left on for several days, as described in the text. At this stage a clamp is being applied to divide the spur Note that the bowel is so placed that the mesentery is not in the spur, and the forceps are arranged so that the bite is at the tip at first.

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[6] In the majority of cases of cancer of the colon a multiple-stage modified Mikulicz operation is indicated In those cases in which an immediate anastomosis can be made the technic here described is applicable only in the almost complete absence of fat and when the tissues are easily accessible and the surface is fairly smooth Otherwise it would be better to strip the stumps of the colon of fecal matter place soft bladed clamps open and clean the ends of the bowel after packing them off well and then suture the mucosa accurately with chromic catgut or silk from within with a continuous suture. After this another row of external sutures may be placed

ogist should carefully examine the mesenteric glands If a number of glands are actually involved, it is well to remove the rectum If the cancer is removed entirely, the procedure is excellent. In ideal resection, the mortality is much greater in rectosigmoid cancer than in cancer of the ampulla In rectosigmoid cancer, obstruction first calls the patient s attention to his trouble by diarrhea In cases in which an emergency colostomy has been done previously, often by the family physician, the operation for rectosigmoid cancer is no more fatal than that for low rectal cancer One should first treat the obstruction by colostomy and, in certain cases, by cecostomy, and wait until the patient has thoroughly recovered Then the radical two stage removal operation should be done as in cancer of the rectum.

Dr Donald C Balfour, Rochester, Minn I am sure we have all appreciated the two points Dr Coffey has made, the first applying to all surgery of the gastro-intestinal tract and the sacrifice of the patient to idealism I think what he had to say about multiple stage operation for such serious conditions as carcinoma, whether in the colon or in the gastro intestinal tract, is exceedingly important. The second thing is what he said about the expense connected with hospital care, and I think his point is well taken. The whole future of the patient is involved a matter of a few weeks one way or the other is really of minor importance

Clinical Notes, Suggestions and New Instruments

AN APPARATUS FOR MASS TREATMENT OF CHILDREN WITH ULTRAVIOLET RAYS *

Benjamin Goldberg M D Chicago

There is a period of about eight months each year during which the amount of ultraviolet rays reaching the ground strata of atmosphere in our large cities is much diminished.

The problem of supplying children with ultraviolet radiation on a mass scale stimulated the building of the apparatus about to be described

The first installation designed was for the Spalding School for Crippled Children, in which a large number of children had bone tuberculosis, it was purchased by the board of education

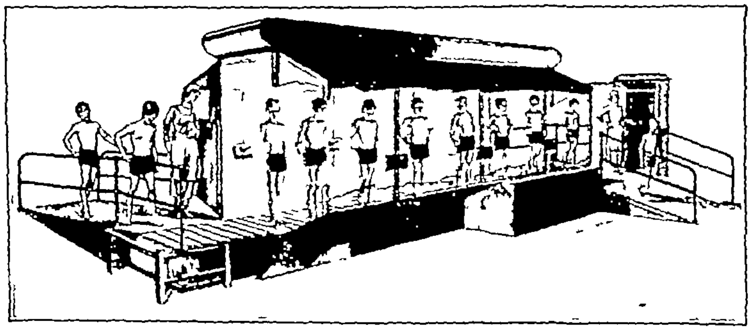

Fig 1 —Artist s sketch with front of apparatus cut away to illustrate operation

A carbon arc unit was first considered The idea, however, was discarded because of the fact that according to city ordinance, each carbon arc unit must have an operator in charge In addition, the amount of heat generated by a carbon arc unit is very large and the treatment period, as time went on, would necessitate long exposure. The length of the exposure would, of course, curtail mass treatment and decrease greatly the number of children who could receive this benefit

As a further objection, the special problem of the crippled children had to be considered It is almost impossible to handle in a mass crippled children with various types of deformities, it would be difficult to move them in and out of the arc of treatment in such a way that they would receive accurate dosage It was decided finally to make use of mercury quartz lamps combined in one unit in such a way as to meet the needs of the crippled children or, indeed, the needs of all school children

With this idea in mind, an apparatus was devised consisting of a sheet metal room, 8½ feet wide, 20 feet long, and 9 feet 2 inches high The "sun room" was elevated about 15 inches from the floor and approached at both ends by ramps, 6 feet in length, graded 2½ inches to the foot. The room is open at each end and a conveyer, on the endless chain principle, runs its entire length.

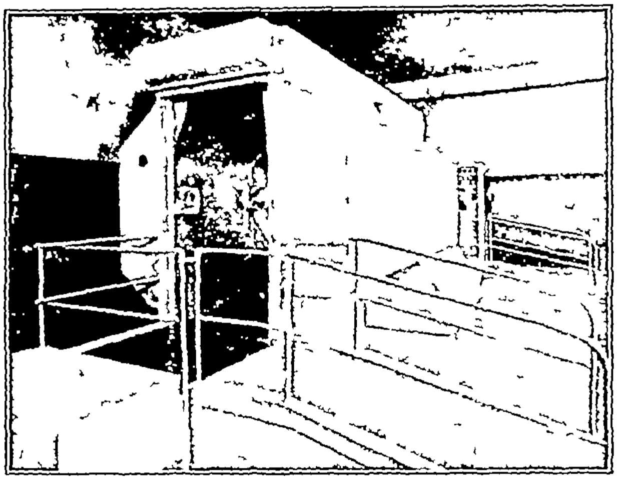

Fig 2 —View of machine constructed and installed at the Spalding School for Crippled Children

This conveyer (on which the children travel) is, by means of the tachometer control, adjustable in speed The rate is controlled by a speed reducing unit which enables the conveyer to travel the length of the chamber at speed variations for the distance of from forty-five seconds to four and a half minutes Running down the center of the conveyer are two heavy black lines about 9 inches apart The children to be treated stand between these lines during their passage through the sun room

On each side of the chamber, or sun room, 2½ feet from the floor are placed three Uviarc lamps, making a total unit strength of six mercury quartz lamps The lamps are supplied with special adjustable reflectors which are constructed to give an even distribution of light at all angles Additional reflection is obtained because of the fact that the whole interior of the chamber is coated with aluminum paint

In order to maintain a comfortable temperature level, exhaust fans have been installed at the top of the chamber These fans promote a breeze of approximately 10 miles an hour and afford a twofold advantage of reducing the temperature and supplying air currents to the skin.

A control board at one end of the unit allows individual control of each arc unit within the chamber and also, by means of the tachometer, permits the necessary regulation of the speed

The intensity of exposure is controlled in three ways

1 Through the speed of the conveyer
2 Through the voltage control transmitted to the mercury arc.
3 Through the curtailing of the number of arcs in use

The method of treatment is simple. The graduated ramp which approaches the entrance will accommodate children on wheel chairs, stretchers or crutches The children are placed between the two lines in their course through the sun room they are kept equidistant from the two sides of the battery units Standing, or in wheel chairs between the lines, they are at a distance of approximately 40 inches from each side.

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* From the City of Chicago Municipal Tuberculosis Sanitarium and the University of Illinois College of Medicine

Depending on the amount of exposure they have already had the children are coursed through the sun room at any indicated speed, varying from forty-five seconds to four and a half minutes They are sent through in groups based on relative sensitivity and are carefully observed for results

It has been found that an exposure of forty-five seconds with the full battery of lamps at a voltage of 75 produces only a very slight erythema in approximately 50 per cent of the children.

As to capacity for early exposure, approximately 250 children can be handled in an hour A nurse stands at each entrance to help the children on and off the conveyer As they go through the room the children wear only a breech cloth and goggles to protect the eyes The apparatus has been in operation only a few weeks at the time of writing A large number of children are being treated and careful data are being prepared for further consideration

104 South Michigan Avenue

BILATERAL FIBROSARCOMA OF THE LOWER EXTREMITIES

C R. Weis M D Dayton Ohio

An American housewife, aged 51, was seen because of bilateral fibrosarcoma of the lower extremities The patient s father and mother four sisters and two brothers were living and well One brother was killed in an accident The patient had had three normal deliveries The diseases of childhood included measles, chickenpox and pertussis She had frequent sore throats At 31 years of age six weeks after the birth of her second child, she had rheumatic fever At the age of 34, a large ovarian cyst was removed At 40, a tonsillectomy was done, but only after joint changes were quite marked At the age of 45, she noticed a swelling beginning on the dorsum of each foot just behind the toe clefts the right slightly larger than the left and both accompanied by occasional sharp, cutting pain No history of trauma or ulcer was obtained The patient consulted a physician who gave her 'vaccine treatments' daily for two weeks Obtaining no relief, she discontinued them The masses enlarged symmetrically and were painful the patient was confined to a wheel chair day and night The pain finally became so intense that she consulted a surgeon who admitted her to a hospital two years before

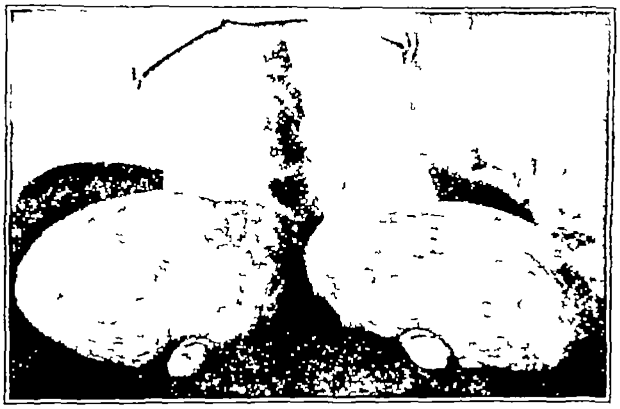


Fig 1 —Gross appearance of tumor masses

Physical examination at this time revealed the feet and ankles incorporated in a large, oval, rather firm mass (fig 1), only the great toe and tip of the second left toe being visible, and having the gross appearance of bread dough that has become dry and cracked. A roentgenogram of the chest showed generalized nodular infiltration A plate of the tumor masses showed bony destruction but no attempt at bone formation The temperature chart showed an occasional rise, while the pulse varied from 80 to 110 The urinalysis showed an alkaline urine The specific gravity was 1 016 there was a slight trace of albumin but no casts The blood count showed red cells, 4 100,000 white blood cells, 9 800, polymorphonuclears 78 per cent small lymphocytes, 16 per cent, large lymphocytes, 6 per cent. The blood Wassermann reaction was negative. Surgery was refused, biopsy was not granted, and the patient returned home with a provisional diagnosis on the hospital record of elephantiasis

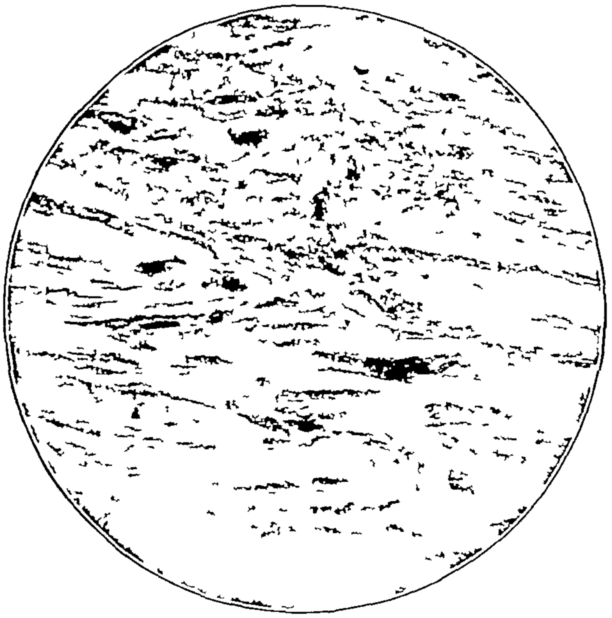


Fig 2 —Microscopic appearance of section of tumor

Three months before her death I was called to the patient's home in the country and asked to relieve her pain At this time the tumor masses had trebled in size and were extending midway to the knee, the contour of the feet and ankles being entirely lost in the tumor mass, with the exception of the heel pads The surface was mainly a gangrenous and necrotic one

At examination the patient appeared emaciated weighing about 90 pounds (41 Kg) She sat in a wheel chair, with her knees spread widely apart because of the size of the tumor masses (136 cm in circumference), she was lethargic and exhausted, coughed frequently and complained of pain in her back, head and particularly the feet The skin and conjunctiva were yellowish brown The mucous membranes, nails and lips were cyanotic. The pulse rate was 120 and very soft The hands showed the characteristic changes of arthritis deformans The left pupil was larger than the right both reacted to light and distance The lips were dry Sordes was present The apex impulse was 11 cm from the midsternal line in the sixth space, 2 cm outside the midclavicular line. The sounds were of the tick-tack variety The blood pressure was 110 systolic, 88 diastolic, and 22 pulse pressure A soft systolic murmur was present at the apex The chest showed many dull areas with increased voice and moist rales in both cases Respiration was 36 The abdomen was distended and tympanic The liver edge was felt 3 cm below the costal margin and was smooth in outline The inguinal nodes were enlarged

Pelvic and rectal examination was not attempted. The temperature range was from 97 8 to 101 3 The urinalysis showed an alkaline urine the specific gravity was 1 008 a trace of albumin was present, there were no casts The blood count showed 3,200 000 red cells, 10 300 white cells and 80 per cent polymorphonuclears The Wassermann reaction was negative. Measures were used to keep the patient as comfortable as possible until she died, Nov 30, 1926

A section of tissue removed post mortem was grayish white, rather firm, with a shiny substance and a brownish thickened and roughened skin covering Sections revealed smooth bundles of fibrous tissue surrounding elongated or spindle-shaped cells with many dilated and congested thin walled blood vessels The skin showed polynuclear infiltration with thickening and increased pigmentation of the skin layer (fig 2)

A diagnosis of fibrosarcoma was made

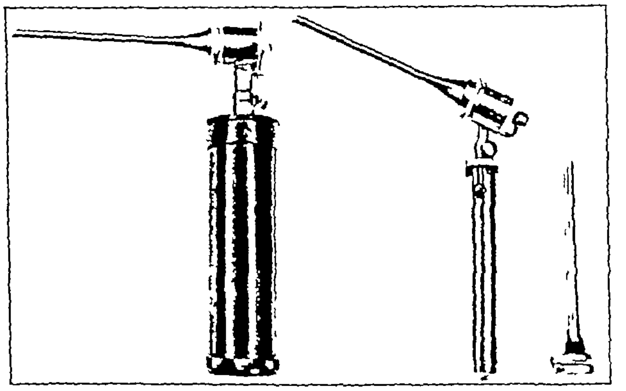
414 Harries Building

A NEW PRACTICAL VAGINOSCOPE FOR USE IN CHILDREN AND VIRGINS*

Samuel K Levy M D Brooklyn
Adjunct Attending Pediatrician and Chief of Pediatric Clinic United Israel Zion Hospital Attending Pediatrician at the Brooklyn Infants Home, Crown Heights Hospital and Shore Road Hospital

After a careful search of the American and foreign literature to date, I have not found recorded any practical electrically illuminated vaginoscope for use in children and virgins I am therefore presenting here my vaginoscope, with my reasons for the measurements adopted

In every female child admitted to our hospitals, infant homes and similar institutions, a vaginal (cervical) smear is made on admission, usually with a cotton tipped wooden applicator I measured a large number of such applicators and found that they varied in size from 13 to 19 French. These measurements were the clue to the proper size caliber tube that I should use in my vaginoscope In deciding on the proper size length of tube that I should use, I met with difficulties Nowhere in the American literature could I find definite measurements as to the length of the child's vagina Ballantyne[1] says, 'The vagina is from 2.5 to 3 5 cm in length in the new-born" No other measurements are given In the Handbuch der Anatomie des Kindes[2] there are given measurements of the length of the anterior and posterior walls of the vagina in children from 1 month to 13 years of age, and these measurements vary from 28 mm to 67 mm, the posterior wall being slightly longer than the anterior Consequently the specifications of my first vaginoscope were for a tube about 3½ inches (8 8 cm) in length and about 18 French. This length of tube will also permit examination of the adult virgin I had the entering end beveled so that the lower surface, which slides along the posterior wall, is slightly longer than the upper or anterior surface, which again conforms to the measurements quoted, and this also permits of greater ease of insertion.



Vaginoscope for use in children and virgins

All else that I need say to complete the description of my invention is that it is an elongated auroscope speculum which can be secured to fit the head of any of the standard battery handles This permits of direct electrical illumination The American Cystoscope Makers, Inc. (Mr Reinhold Wappler) and the Welch Allyn Company, have been kind enough to make and present me with the apparatus shown in the accompanying illustration As can be seen, a small magnifying end piece such as now fits on the auroscope head piece, is a useful part of this combination This will permit of the passing of an applicator through the tube (For those who desire additional safety in introducing the instrument an obturator may also be secured) I am assured that both the vaginoscope end piece and the magnifying lens can be secured for a reasonable sum

In the past such instruments as urethroscopes, cystoscopes and nasal speculums have been used for vaginal examinations in children and virgins I claim the following advantages for my vaginoscope over other apparatuses formerly in use reasonable cost, ease of manipulation, direct electrical illumination and magnification, universal use of the electrical auroscope with ease of attachment of my vaginoscope end piece and magnifying lens, and easy sterilization It has the advantage of being not only a great aid in treatment with direct vision of the parts but also the ideal way of obtaining vaginal and cervical smears By examination of the interior of the vagina, foreign bodies that may be an underlying cause of prolonged vaginal discharges may be seen and readily removed It may also be of value in cases of suspected rape I am sure additional advantages will become manifest with its future use I have not observed any ill effects from its use in a series of children examined in private practice, at the hospital and at the Brooklyn Infants Home, and varying in age from about 3 months to 14 years My observations of these and other children will be the subject of a future paper

1694 Nostrand Avenue

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* Abstract of a paper read before the New Utrecht Medical Society May 27 1929

[1] Ballantyne in Abt's Pediatrics Philadelphia W B Saunders Company 1: 370 1923

[2] Handbuch der Anatomie des Kindes part 1 2 90 1927

GLANDERS

Ralph W Mendelson M D Albuquerque N M

Glanders is not common in man although it is probable that many cases are not recognized because of the protean manifestations of the disease, with special reference to the skin lesions Strictly speaking, the term glanders is applied to the infection with *Bacillus mallei* as it affects the nasal mucous membrane and the internal organs The term farcy is used to designate the lesions in the skin and lymphatics Generally speaking, glanders may be defined as a specific infectious disease due to *B mallei*, common in the horse, mule and ass, contracted by man accidentally either through skin or nasal mucous membrane inoculation

The two following cases illustrate the course as a chronic skin and mucous membrane infection

REPORT OF CASES

Case 1—A white woman aged 36 who was reared on a farm and whose previous history was irrelevant except that she came into intimate contact with two horses that died of an unknown, but suspicious, disease eighteen months previous to her present examination first noticed a slight nasal discharge and shortly afterward there appeared a small vesicle near the tip of the nose The nasal discharge gradually increased and became mucopurulent, while the skin lesions advanced until the entire upper lip and nose presented an ulcerated, discharging surface She consulted a number of physicians and the diagnosis made was either syphilis or epithelioma At no time did she suffer any marked constitutional symptoms At the time of examination the patient was well nourished and the physical observations were negative except as portrayed in figure 1 The temperature, urine and blood counts were all within normal limits The blood Wassermann reaction was negative The discharge from the nose was profuse There were no laryngeal symptoms The skin lesions were covered with a thick, yellowish crust which after removal presented numerous isolated ulcerations Smears from the nasal discharge and from the ulcers showed a heavy infection with a gram-negative, slightly curved rod. This organism proved to be *B mallei* and the patient's blood agglutinated a foreign organism in high dilution

Treatment with an autogenous vaccine was instituted together with such local treatment as was indicated but the patient was not under observation sufficiently long for the effect of such medication to be determined

The diagnosis in this case depended on the epidemiology, the characteristic nasal discharge, the negative Wassermann reaction, the finding of a suspicious looking organism in the discharges, the confirmative laboratory observations with regard to this organism, and the positive agglutinating properties of the patient's blood Special reference is made to the epidemiology as indicating the probable source and nature of the infection

Case 2—A Mexican youth aged 15 presented himself for treatment, because of the loss of sight in his left eye, a severe infection in his right eye, a profuse mucopurulent nasal discharge, and extensive ulceration of his nose and upper lip as illustrated in figure 2 His previous history is negative except that he lived on a small ranch and came in intimate contact with a particular horse that died of an unknown disease The patient insisted that about a year and a half before he first noticed a discharge from his left eye This was followed by a nasal discharge and ulcerations of the tip of the nose that gradually spread to the upper lip and involved the greater portion of the external nose. He next noticed some difficulty in his throat, a slight hoarseness in his voice combined with some slight difficulty in swallowing About six months previous to his present examination his right eye became infected He did not complain of any marked constitutional symptoms at any time. On physical examination he presented an emaciated body, considerable enlargement of all of the glands in the neck, a normal temperature, a normal blood count, normal urine and a negative blood Wassermann reaction He could speak only in a whisper The right eye presented considerable corneal ulceration with extensive conjunctival inflammatory reaction The discharge was not profuse. The left eye showed slight corneal ulceration with considerable conjunctival reaction and profuse mucopurulent discharge. The nasal passages were blocked from encrusted discharge The skin lesions were covered with a thick, yellowish encrusted mass that involved the mucous membrane of the upper lip to the gum line The throat lesions were subacute. The tonsils were not affected, the palate was not involved. The larynx was injected There were no ulcers visible

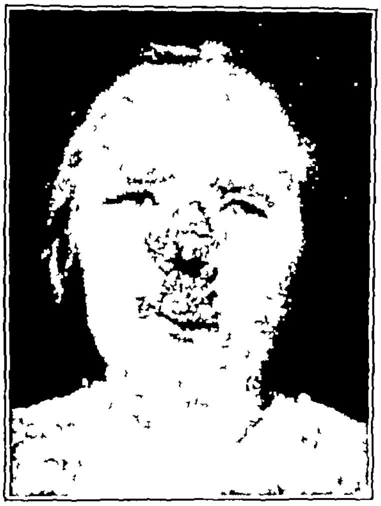

Fig 1 (case 1) —Ulcerations covered with crust.

From the lesions, eyes, nasal mucous membrane and skin ulceration was obtained a mixed infection consisting of *Staphylococcus aureus* a diplococcus and many gram-negative, slightly curved rods with irregular staining properties, that proved to be *Bacillus mallei* The blood agglutinated a foreign organism in high dilutions

The diagnosis depended on the epidemiology, the characteristic nasal discharge the negative Wassermann reaction, the finding of a suspicious looking organism in the lesions, the confirmative laboratory observations with regard to this organism and the positive agglutinating properties of the patient's blood. The case is interesting in view of the initial lesion in the right eye. Although the epidemiology is not positive it may be assumed that the intimate contact with a horse that died of an unknown disease was the probable source of infection. It was not possible to obtain a history with regard to any of the other live stock.

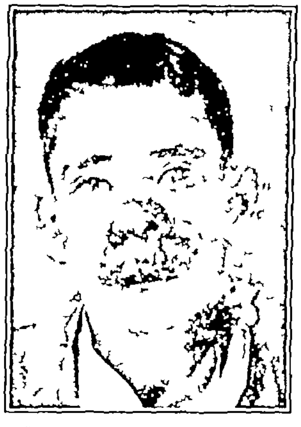

Fig 2 (case 2) — Extensive ulceration of nose and upper lip

The treatment consisted of a mixed autogenous vaccine given only once a week as it was not possible for the patient to come to the city more often Local treatment consisted of "hot-toweling" the encrusted ulcerations until the scabs had been removed and then applying a 20 per cent mercury ointment

The results have been extremely satisfactory The throat lesions have cleared up The voice is almost normal The infection in the right eye has disappeared and the nasal discharge has practically ceased The glandular enlargement is the same. Four weeks after the use of the vaccine, *B mallei* could not be demonstrated in the discharges and the only organism present in the nasal discharge was a diplococcus with an occasional *Staphylococcus aureus* The patient has gained 3 pounds (1 4 Kg) and states that he feels materially improved The sight in the right eye is not affected. The sight in the left eye is lost The skin ulcerations have practically healed and the inflammatory reaction has been reduced 75 per cent.

COMMENT

The diagnosis in these cases is suggested by the epidemiology and the characteristic nasal discharge. In these two cases direct smears showed a profuse infection with a suspicious organism that proved to be *B mallei* The skin lesions might be confused with syphilis or epithelioma, and I have seen many cases of gangosa in the tropics which resemble chronic glanders. In syphilis the positive Wassermann reaction would clear up the diagnosis if the bacteriologic observations were negative. Epithelioma would be determined by tissue examination and gangosa in the tropics by the therapeutic test with arsenic, immediate results follow treatment.

The prognosis is not good Complete eradication of all the signs and symptoms is not proof of a cure. The disease may remain latent for long periods and a sudden acute exacerbation terminate fatally In view of the results obtained by vaccine treatment it would seem advisable to keep these patients under observation for many years, stimulating their resistance by the use of vaccines and proper hygiene measures

608-609 First National Bank Building

MIDLINE PRESTERNAL ULCER FREQUENTLY A GUMMA *

Owen H Wangensteen M.D Minneapolis

No diagnostic criterion is infallible Occasionally the most dependable symptom or sign may be absent When the cardinal signs of a given disease are present, the diagnosis is usually arrived at with facility When the nature of an ailment remains obscure because of the absence of the classic occurrences peculiar to that disease, diagnostic criteria often thought to be of lesser import attain an unusual significance.

In his "Introduction to Surgery," Rutherford Morison[1] mentions a diagnostic sign of gummatous ulcer that merits more serious attention by physicians in the differential diagnosis of such ulcers Syphilitic ulcers he says, choose the midline where the blood supply is normally the least

In the case reported here, a diagnosis was arrived at solely through the presence of an ulcer in the midline over the sternum, suggesting the possibility that the ulcer as well as the major complaint of pain and ulceration in the rectum for which the patient had sought relief for fourteen years might be due to syphilis It is my purpose in this paper to direct attention to a valuable but little recognized earmark of a disease that falls within the province of every practicing physician.

REPORT OF CASE

Mrs M L, aged 37, admitted for the first time to the University Hospital, Jan. 12, 1928 because of a discharge of blood and pus from the rectum for the past four years had had considerable pain in the rectum for fourteen years at times so severe as to be incapacitating In 1915, hemorrhoidectomy was done, followed by recurrence of the hemorrhoids in 1917 In 1923 the patient sought electrical treatment because of the pain in the rectum Meanwhile she had had conservative treat ment by diet, enemas and local applications of various sorts without relief A diagnosis of ulcerative proctitis was made at this time and, because of the failure of conservative treatment a colostomy was advised and accepted by the patient After this operation the patient was definitely better, but the pain in the rectum continued

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* From the Department of Surgery of the University of Minnesota Medical School

[1] Morison Rutherford An Introduction to Surgery Bristol John Wright & Sons 1910 p 35

In November, 1927, a fistula developed beside the anal opening and there had since been considerable discharge of blood and pus from the rectum

On admission the patient appeared well developed and well nourished The general physical examination was essentially negative except for a double barreled colostomy in the left inguinal region and a fistula that extended from the posterior portion of the left labium to establish a communication with the rectum. There was pronounced stenosis of the anal canal, the walls of which were indurated and irregular On digital examination of the rectum, the patient complained of considerable pain. Posteriorly, there was a fistula running beneath the sphincter into the rectum The anal canal was almost entirely covered by numerous shallow, ragged ulcers There was almost complete absence of induration in the surrounding tissues.

Routine examination of the blood and urine gave normal results The blood Wassermann reaction was negative Specimens taken from the ulcers revealed chronic inflammatory change. Scrapings from the ulcers failed to reveal parasites and guinea-pig inoculation tests were negative. There were no parasites in the stools

The diagnoses were (1) ulcerative proctitis, (2) multiple anal fistulas, and (3) old colostomy

Various types of irrigations, and treatment with soft x-rays and the ultraviolet ray had no influence on the lesions During her stay in the hospital the patient developed a shallow ulcer about 38 mm in diameter, with its center slightly to the left of the midline over the sternum at about the level of the third rib The margins were bluish and very ragged, and about the periphery of the ulcer the skin exhibited slight brownish pigmentation The edges were slightly undermined and the floor was covered by bluish red exudate. There was no induration in the surrounding tissues January 30, the fistulas were laid open and the anal sphincter was divided No material change resulted and the patient was discharged, February 27

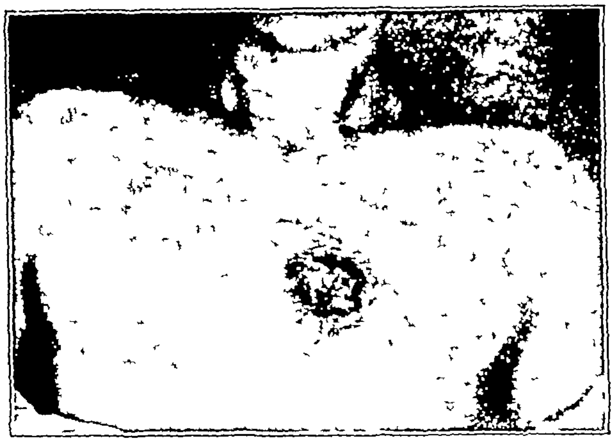

Fig 1 —Shallow ulcer over the sternum almost round with irregular margins slight pigmentation in the peripheral healthy skin. The ulcer itself is not typical of a syphilitic ulcer

The patient was readmitted, May 25 1928, because of pain and discharge with her condition essentially unchanged At this time a blood and urine examination were again negative The Wassermann reaction of the blood and spinal fluid was negative The Nonne and Noguchi tests were negative, and the intraspinal pressure was 30 mm. of water There were three cells per cubic millimeter Neurologic examination was negative The patient was also seen on several occasions by the consulting dermatologic staff

Reexamination of specimens from the ulcers about the anus and chest again revealed only chronic inflammation No parasites could be found in smears from either situation. Irrigations with surgical solution of chlorinated soda were instituted but had no apparent benefit and were abandoned Saline irrigations likewise did not have a demonstrable effect. There was no improvement following ultraviolet irradiation. September 2, quinine packs were applied to both ulcers every three hours No change was noted after ten days

I saw the patient for the first time, September 11, and felt that despite the absence of serologic evidence of syphilis the midline shallow ulcer over the sternum with very little change in the adjacent skin was strongly suggestive of syphilis The lack of *induration* about the anorectal lesion was also most unusual A saturated solution of potassium iodide in doses of 2 cc was given three times a day Within a week there was definite improvement in the ulcer over the chest with beginning healing at the margins September 20, a weekly administration of 45 Gm. of neoarsphenamine was begun. By the end of the month there was definite epithelization of the skin margins of the anorectal lesion, and, October 15, the ulcer over the sternum was entirely covered by epithelium November 3 the patient was dismissed to attend the outpatient dermatologic clinic for further antisyphilitic treatment.

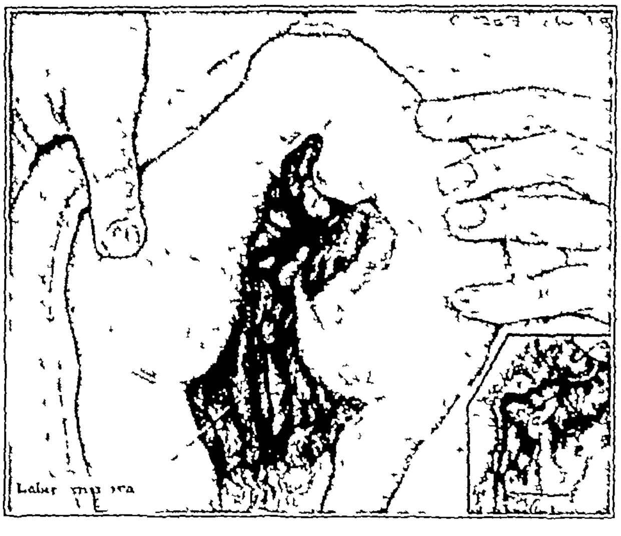

Fig 2 —Appearance of anorectal lesion for which patient sought relief two weeks after the administration of potassium iodide was commenced Definite evidence of healing at the periphery is present. This lesion healed much more slowly than did the presternal ulcer Inset *a* ulcerating areas *b* rectal cloaca *c* remnants of anal sphincter *d* rectovaginal septum (covered with granulation tissue) *e* granulation (covered with new epithelium) *f* vaginal orifice.

November 7 the patient was readmitted for the treatment of a severe arsenical dermatitis following the administration of neoarsphenamine. Under the influence of daily intravenous injections of sodium thiosulphate, given under the direction of Dr Henry Michelson of the dermatologic staff, the patient improved and was dismissed, December 13 Prof Edgar Brown of the department of pharmacology demonstrated the presence of arsenic in the urine

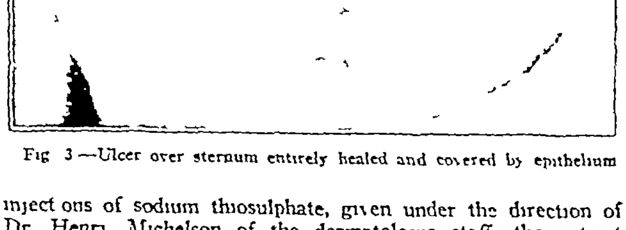

Fig 3 —Ulcer over sternum entirely healed and covered by epithelium

The lesion over the sternum has remained well healed and the lesion in the anorectal area is also healed Occasionally there is slight bleeding from the rectum, but digital and visual examination of the rectum with the proctoscope fail to show local persistence of disease

Because of the contraction of scar tissue about the rectum, the external opening is somewhat stenotic, which in all likelihood satisfactorily accounts for the occasional discharge of blood brought about by the accumulation of mucus in the lower bowel As a consequence of the loss of the sphincters of the rectum, the complete restoration of the continuity of the bowel is not feasible

Serologic tests for syphilis have still continued negative Since the patient's dismissal from the hospital, a perforation in the nasal septum typical of syphilis has developed Therapeutic test as well as the latter occurrence therefore give credence to the belief suggested by the midline ulcer that the anorectal lesion was due to syphilis

COMMENT

Some years previously in another clinic I saw a similar presternal midline ulcer which was being excised under the impression that it was malignant Histologic examination of the removed tissue showed evidence of chronic inflammation and the presence of giant cells which appeared to warrant the diagnosis of tuberculosis Reference to the chart revealed that the patient had active syphilis with a positive Wassermann reaction and syphilitic periostitis of both tibias Despite these conditions there had been no intimation of a suspicion that the ulcer over the sternum might be a gumma, which it undoubtedly was That gummas in skin have no pathognomonic histologic picture is well known and the occurrence of giant cells in such lesions is not infrequent In the absence of the cardinal signs of syphilis, the presence of a midline ulcer should arouse a suspicion of syphilis

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VARICOSE VEIN OCCLUDER IN THE INJECTION TREATMENT OF VARICOSE VEINS

Frank V Theis M D Chicago

While in Vienna recently I became acquainted with the use of a simple and practical instrument the varicose vein occluder With its more efficient technic during and following the injection treatment of varicose veins, the percentage of satisfactory results will be greatly increased The routine procedure that I have observed since my return is little more than an intravenous injection with digital compression of the main vein Naturally, the frequent failures to produce complete thrombosis with the 50 or 60 per cent sugar solutions have led to the substitution of more powerful and dangerous chemical irritants Hypertonic salicylate, sodium chloride, quinine and urea hydrochloride and other solutions are now commonly injected. I believe that the improved technic that I will presently describe will be of great benefit in eliminating many of the failures previously mentioned

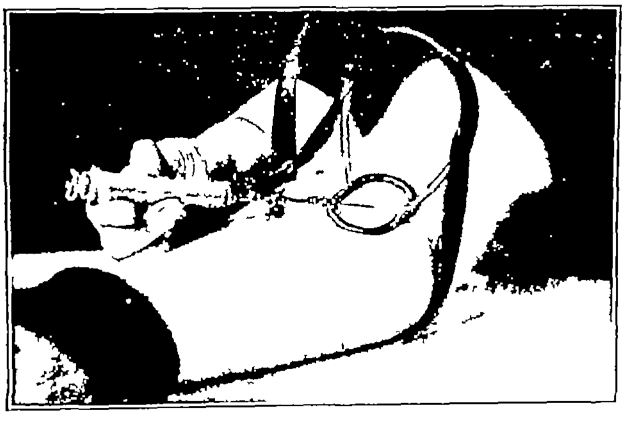

Fig 1 —Self retaining varicose vein occluder and special two-way stopcock needle connection

The varicose vein occluder applied with moderate pressure acts to obliterate all the anastomotic superficial veins to the area to be injected The undiluted concentration of the weaker sugar solutions is sufficient to produce the necessary changes for thrombosis Minimizing the blood dilution of the injected irritant is essential only when the less harmful solutions are used. Lack of thrombosis and the subsequent substitution of other chemicals can be attributed to this factor alone. The stirrup type of occluder (fig 1) with or without the leather strap attachment originated in Riehl's varicose vein clinic at the Allgemeine Krankenhaus in Vienna In clinic or dispensary work in which assistants are available, the modification (fig 2) with the single instead of the loop handle permits a greater range and ease for the insertion of the needle

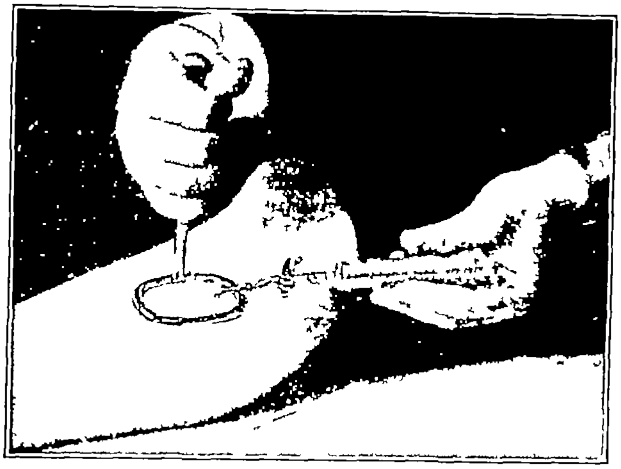

Fig 2 —Modification for use when assistance is available.

The following technic is employed The patient stands on the floor while the group of veins to be injected is selected In order to break the weight of the column of blood, the obliteration of groups of vessels at the knee or lower thigh is first desired The area chosen for injection is then marked with iodine and the occluder applied as the patient is lying on the table A 10 cc syringe with a two-way stopcock needle attachment is used both to allow the blood to escape and for the injection Digital pressure on the tissues within the loop of the occluder will hasten the more complete emptying of the vessels Some difficulty may be encountered in producing a bloodless area because of the presence of deep communicating branches, but these are of far less concern then the superficial anastomosis, which is taken care of by the occluder The turning of the stopcock will now permit the injection of the chemical irritant into the bloodless veins, where it is allowed to act on the intima for from three to five minutes Removal of the occluder at the end of that period practically assures one of subsequent thrombosis even when the most innocuous 50 or 60 per cent dextrose or levulose-dextrose solutions are used.

The advantages of this method are manifest Some attempt is made to have the veins as bloodless as possible and some attempt to hold the irritant at its maximum concentration for sufficient time to assure adequate intima changes for thrombosis to follow In the Vienna clinic the large number of injections over a period of years have given almost uniformly successful results with the routine levulose-dextrose solution as the chemical irritant The varicose vein occluder is of great practical value in the efficient handling of varicose veins by means of the injection method

Presbyterian Hospital

Diagnosis—There are few diagnostic signs in medicine To reach a diagnosis demands careful exercise of our powers of reasoning We must proceed in an orderly fashion and without preconceived ideas He who begins his examination by looking at an x-ray plate is very likely to lose his way in his search for an explanation of some unexpected shadow with regard to which he had formed a preconceived idea He should not approach his x-ray plate before he has acquired that other evidence which he gains from a thorough physical examination. —Thayer, W S *J Tennessee M A* October, 1929

NONSURGICAL TREATMENT OF A PILONIDAL CYST

Edgar R. Maillard M.D., New York

It is generally recognized that certain types of acquired sinuses and fistulous tracts, resistant to the more ordinary forms of treatment, have been successfully healed by means of the galvanic current. Since none of the cases of pilonidal cysts (sacral fistulas) reported in the literature have been treated by this method, an account is given here of such a case. Pilonidal cysts (sacral fistulas) are considered to be developmental anomalies and have been successfully healed only by complete excision of the tract or tracts. Other methods of treatment, such as incision or cauterization with chemicals, have accomplished only temporary relief.

REPORT OF CASE

History and Examination.—A hairy, thick set, dark complexioned white man, aged 22, had had a troublesome discharge occurring near the anus for about six months. A year previously he had experienced the same kind of discomfort, but at that time the discharge stopped without any treatment. There was little pain present. The patient's general health was only fairly good, he tired quite easily and had experienced several attacks of furunculosis, the furuncles appearing chiefly on the arms, legs, buttocks and scrotum.

Examination of the region above the anus with the gluteal folds separated showed two oval openings about one-eighth inch in diameter and about 1 inch apart, with hair protruding through both of these openings. They were situated in the midline over the region of the sacral bone. When probes were inserted through the openings the tracts were found to be separated for a short distance by tissue which was unhealthy in appearance. The direction of the probes indicated that the tracts had converged and were found to be confluent, extending in almost to the sacral bone. Pressure caused some pain and a thin brownish fluid was expelled. A complete physical examination did not reveal any other abnormalities and roentgen examination of the pelvis showed no pathologic changes in the sacral bone.

Treatment.—A copper electrode attached to the positive pole of the galvanic current was introduced successively into the sinuses. The negative pole was attached to a pad moistened with saline solution and placed on the patient's back. The current was turned on and gradually increased until 20 milliamperes was registered on the milliamperemeter. Each treatment was continued for twenty minutes, at first at five day intervals, later at seven, and finally at ten day intervals. The entire series required about three months.

The treatments caused a great deal of destruction with exfoliation of the disintegrated material containing hair and other cellular debris. The discharge became more profuse, particularly for the first two days following each treatment. The intervening unhealthy tissue was completely destroyed, one common opening being formed extending almost to the sacral bone. As the destruction became complete the intervals between treatments were lengthened to allow more time for healing. The tract became lined with a healthy granulating tissue which bled quite easily when the copper electrode was introduced. Consequently electrodes of smaller size were used with each subsequent treatment until healing was complete. The new tissue was soft and pliable, resembling closely the texture of the normal skin.

Result.—There has not been any recurrence of the condition after six months and no attacks of furunculosis have occurred. The patient has gained weight and feels generally improved in health. At no time was he incapacitated from his occupation by the treatment or inconvenienced by pain, though a local anesthetic might have been used if it had been indicated.

SUMMARY

A case of pilonidal cyst was successfully healed by means of the galvanic current without pain and without incapacitating the patient.

167 East Sixty-First Street.

Council on Pharmacy and Chemistry

REPORTS OF THE COUNCIL

The Council has authorized publication of the following report.
W. A. Puckner, Secretary.

TOXOGON NOT ACCEPTABLE FOR N. N. R.

Toxogon is the therapeutically suggestive name applied by the Von Winkler Laboratories, Inc., Chicago, to a preparation proposed for the treatment of infectious diseases, particularly gonorrhea. In the information submitted to the Council, "Ricin Argen" is given as a synonym. According to the information furnished, "Toxogon consists of silver bound in colloidal form upon Ricinic acid." The following indefinite method of preparation is furnished: "TOXOGON is manufactured by introducing silver into a suitable colloid and introducing the silver colloid into ricinic acid and transferring the insoluble salts through suitable chemical agents, such as bases, into soluble staple [stable?] compositions." Definite information with regard to the composition of Toxogon was not furnished; tests and standards for its control were not supplied, nor was the percentage of silver contained in the compound declared.

In the advertising for Toxogon which has been sent to physicians, the synonym Ricin Argen is replaced by "Sulpho Recin Argen" and the preparation is stated to be "Monosulforicin Silver."

It is claimed that Toxogon has the properties of silver compounds but that the combination with ricinic acid "lowers the surface tension of the solution to a higher extent than is possible with any other agent and permits a much freer permeation" and that "Laboratory tests on vial cultures of streptococci showed a one (1) minute efficiency of 1% dilutions"—whatever that may mean. In the advertising sent to physicians the extreme claim is made that "It represents the most ideal antiseptic and germicidal solution and for this reason is especially adopted in the treatment of infectious diseases." No evidence in support of these claims was furnished the Council nor was any evidence submitted or contained in the advertising which compares the efficacy of this preparation with the many well known silver preparations that are official in the U. S. Pharmacopeia or that have been accepted for New and Nonofficial Remedies.

The Council found Toxogon unacceptable for New and Nonofficial Remedies because its composition is not adequately declared, because no evidence is available to indicate that the composition and uniformity of the product is controlled, because the claims advanced for it are unwarranted in the light of the available evidence, and because it is marketed under a therapeutically suggestive name.

When the preceding statement was submitted to the Von Winkler Laboratories, a reply was received which stated the silver content of the preparation but which did not furnish definite information as to the form in which this is present. It does not remove the element of mystification as illustrated by the meaningless formula $[Ag(NH_3)+2]$. If a guess may be ventured it is probably a suspension of colloidal silver in a castor oil ammonia soap solution. In the firm's reply, much was made of the surface tension of the preparation, but no direct evidence of superior penetrating power of the preparation is furnished and it is most improbable that the presence of ammonium ricinoleate would enable colloidal silver to penetrate even through dead membranes, much less through living tissues. As evidence for the superior value of Toxogon, the reply submitted reports from two physicians. One stated simply: "I take great pleasure in stating to you that I have used your product Toxogon and found it as represented efficient, and satisfactory in every respect." The second included meager clinical reports which do not present any comparison with other silver compounds. The Council therefore affirmed the rejection of Toxogon for the conflicts stated in the report.

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SATURDAY, NOVEMBER 2, 1929

THE PRESCRIBING OF NARCOTICS

Narcotic addiction receives a vast amount of attention both in the medical and in the lay press. Many physicians and sociological investigators are convinced that the amount of attention is beyond the requirement in view of the number of persons involved or the scope of the problem. Nevertheless the life of the narcotic addict is tragic and his existence difficult for himself and all who are associated with him. More and more the psychologists are convinced that mental rehabilitation is perhaps the most important step in the control of the addict. As pointed out in the report of Mayor Walker's Committee on drug addiction, it is necessary to provide the addict with some means of earning his support and to develop in him a state of mind which will make him earnest, cooperative and honestly desirous of being permanently free from his habit. Many, perhaps all, addicts are psychopathic and oppose any effort at rehabilitation. Indeed, it has also been suggested that one of the most important steps in cleaning up narcotic addiction would be to divide all addicts into two groups—those with whom something might be done in the way of restoration to useful existence, and those whose records give little promise of a successful result.

For years there has been much debate as to the causes of narcotic addiction. Again and again it has been urged that physicians are responsible for initiating such habits through the careless prescription of drugs over long periods of time. It is well recognized, however, that there are other sources of narcotics beyond the medical profession. There has been for years an underground traffic in morphine, cocaine and their derivatives. The profits of distribution have been sufficiently great to attract into this commerce masterminds of the criminal classes. Unfortunately, in some of our states the prescribing of narcotics is not limited to the medical profession, but osteopaths and other practitioners who have had little or no study of the pharmacology and proper use of such remedies are granted the right to prescribe them. Quite certainly California, Indiana, Maine, Massachusetts, Michigan, Minnesota, Nebraska, Texas, and possibly Kansas regulations permit osteopaths, under some circumstances, to use and prescribe narcotic drugs. The Harrison law has, no doubt, served the salutary purpose of limiting to some extent the traffic in narcotics, but, as long as this type of prescribing is permitted, the limitations can be of little avail.

In every attempt to find out the most important drugs prescribed in medicine, opium and its derivatives have been included among the first five. These remedies are potent. In proper dosage they invariably secure the effects for which they are indicated. Their very potency should, however, be kept constantly in mind in every prescription. As with all potent remedies, they should be given only to secure certain effects. It is, furthermore, of the utmost importance to remember that only a sufficient amount of the drug should be given and only a sufficient number of doses to secure the effects desired and that the drug should be promptly discontinued immediately thereafter. This is in accord with the best tenets of the individual practice of medicine.

It would be preposterous to say that physicians have never been responsible for the development of drug addictions in some of their patients. It would be equally preposterous to say that physicians have been responsible for the vast majority of cases of drug addiction. Unquestionably the nature of the patient, his personal psychology, his social and economic problems are, not infrequently, far more responsible than any error on the part of the doctor who attends him for his lapse into slavery to the narcotic. The physician should, of course, have in mind these factors. If he does so, he is much less likely to be associated with the development of addiction than when he takes the chance of prescribing purely to secure relief from pain.

Opium and morphine are among the oldest of the drugs that may be used to secure sleep and relief from agony. Within the past quarter of a century there have become available a considerable number of synthetic preparations, particularly coal-tar derivatives and preparations of barbital, which are useful in a similar way. Not infrequently the proper prescribing of such a preparation, singly or in combination, permits the avoidance of the prescription of the more habit forming narcotic.

The furor and the turmoil associated with recent considerations of narcotic addiction, the propaganda for legislation to limit the prescription of narcotics, the discussions in leagues and societies which have made narcotic control their special problem, are leading unquestionably toward the time when attempts may be made to proscribe the use of such drugs except in specifically limited instances, if not altogether. The insufficiency of enforcement of present legislation prompts not infrequently new schemes for enforcement and new methods of raising revenue to provide the means In almost all such plans, increasing assessment of the cost of operation on the medical and pharmaceutic professions, which are chiefly concerned in the prescribing and dispensing of narcotics, has a prominent place Obviously such costs should be, if they are not usually, passed on to the consumer—that is, the public, however, a direct assessment of the consumer would be more proper from every point of view The responsibility rests directly on the medical profession to use these drugs only in instances in which they are quite certainly indicated and only for such periods of time as they may absolutely be demanded by the nature of the case To abolish their use in medicine or to inhibit their use too greatly because there exist among our people defective and criminal classes by whom the use of narcotics is grossly abused would be contrary to the principles of science and justice, which should govern the affairs of intelligent men

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PHYSIOLOGIC HOMEOSTASIS

Half a century ago the French physiologist Claude Bernard wrote that all the vital mechanisms, however varied they may be, have only one object, that of preserving constant the conditions of life in the internal environment By the latter was meant that entity which has been described as the fluid matrix of the body In spite of a continued succession of influences that tend to bring about changes in the fluids and tissues—for example, hot or cold environments that tend to heat or chill the organism, or stimuli that provoke vigorous action and bring about oxygen want—the tendency to readjustment so that characteristic equilibriums or "steady states" will be restored is always in evidence For this general idea of uniformity or stability in the organism, Cannon[1] of Harvard University Medical School has proposed the designation "homeostasis"

Physiologic homeostasis is already known to involve many biologic features They include body temperature, hydrogen ion concentration, and the osmotic pressure of the cell environments, they also involve the materials specifically needed for cellular function, including the proximate principles, water, inorganic elements, oxygen and perhaps specific hormones having general and continuous effects The disruption of the organization and influences by which these are maintained in homeostatic relationships may present the picture of disease, accordingly the problems involved are of great interest in medicine The advantage of this conception lies in the points of view that such a formulation tends to develop

In a scholarly review, Cannon[2] has distinguished some of the general types of homeostasis which involve the regulation of supplies in the body and the control of physiologic processes The former includes, for example, the storage and depletion of water Just how the water is brought to the reservoirs, how it is held there, and how it is released as required for preserving the osmotic homeostasis of the blood, is not yet satisfactorily explained The solution of this problem would throw much light on the pathology of the accumulation of water as it is seen clinically in edema, likewise of the desiccation characteristic of anhydremia The preservation of the sugar level of the blood whereby at one extreme glycosuria is avoided, and at another hypoglycemic shock, affords an added indication of the manifold significance of homeostasis The homeostasis of the blood proteins and the agencies that control storage or release of the albuminous components are as yet largely a matter of conjecture Cannon has pointed out that storage of materials in the body may be looked on as a regulatory mediation between supply and demand Back of storage, however, and assuring provisions which can be stored, he adds, are powerful motivating agencies—appetite and hunger and thirst Because of pleasurable previous experiences with food and drink, appetites invite to renewal of these experiences, thereby material for the reserves is taken in If the reserves are not thus provided for, hunger and thirst appear as imperious stimuli The neutrality regulations of the body have long been stressed in the sense of a homeostatic adjustment

At a time when purely mechanical and chemical reactions have attained a sort of exclusive popularity in the interpretation of biologic phenomena, it becomes interesting to consider the functions of the nervous system in relation to physiologic adjustments Through the cerebrospinal, or voluntary, nervous system man becomes acquainted with and reacts to his external environment Every response of this system, even the slightest, Cannon[3] has reminded us, is associated with the using up of oxygen and energy-yielding material, and at the same time with the production of waste Consequently, the activities of this system are continuously producing changes in the fluid matrix of the body In order that the internal environment shall be maintained in a uniform state, external activity must necessarily be mirrored in an internal adjustment Otherwise expressed, for constancy of the fluid matrix, therefore, every move in relation to the outer world must be attended by a rectifying process in the inner world of the organism According to Cannon, the cerebrospinal nervous system, responding to external stimuli, may be called the exterofective system, and the autonomic system, affecting the domestic affairs of the interior of the organism, may be called the interofective system. When activity of the exterofective system disturbs the fluid matrix of the body, it is the function of the sympathetic division to maintain homeostasis by its interofective functions. In accordance with this principle, during the performance of vigorous muscular work some of the most conspicuous concomitant changes—the liberation of sugar from the liver, the pouring out of sweat, the dilatation of the bronchioles, the contraction of the splanchnic area, the acceleration of the heart and the discharge of corpuscles from the spleen into the general circulation—are brought about through the intermediation of the impulses along the sympathetic division of the autonomic nervous system. They tend toward homeostasis. Medical research is destined to ascertain the homeostatic agencies in the body that prevent disorder. As Cannon[2] has summarized the outlook, effective methods of attaining homeostasis are significant in comparison with the methods in systems in which steady states are not yet well developed, the regulation of homeostasis in higher animals is probably the result of innumerable evolutionary trials, and knowledge of the stability which has finally been achieved is suggestive in relation to the less efficient arrangements operating in lower animals and also in relation to attempts at securing stability in social and economic organizations. Finally, continued analysis of biologic processes in physical and chemical terms must await a full understanding of the ways in which these processes are roused to perform their service and are then returned to inactivity. Indeed, regulation in the organism is the central problem of physiology.

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[1] Cannon W B Physiological Regulation of Normal States Some Tentative Postulates Concerning Biological Homeostatics Jubilee Volume for Charles Richet Paris, 1926 p 91

[2] Cannon W B Organization for Physiological Homeostasis Physiol Rev 9: 399 (July) 1929

[3] Cannon W B The Sympathetic Division of the Autonomic System in Relation to Homeostasis, Arch Neurol & Psychiat 22 282 (Aug) 1929

VITAMIN D AND MILK

In experimental animals receiving food decidedly unsatisfactory in its calcium and phosphorus content as well as deficient in vitamin D, the administration of cod liver oil or viosterol (irradiated ergosterol) results in striking physiologic improvement, whereas most natural food materials are too low in vitamin D to exert similar prophylactic or curative effects against rickets developing on these drastic diets. The effort is made in most quantitative nutrition experiments to vary only the factor under consideration and to provide a basal diet otherwise adequate. But heretofore in vitamin D studies the diets have often been inadequate in mineral as well as in vitamin content. For several years Osborne, Mendel and Park[1] have been engaged in formulating a "synthetic" diet that will permit the study of changes in the development of the bony structure as a result of alterations in a single factor of the food. They use purified dietary ingredients and a salt mixture which can be maintained comparable except for a single element and which can be altered to make the diet vary in its potential acidity or alkalinity. By the use of their basal diet the vitamin supply, the protein level, the modifying effect of fats and carbohydrates respectively, the potential reaction, the ratio of the bone forming elements to one another and the relative "levels" at which they occur in the intake can be studied independently or conjointly. This diet should be useful in many types of nutrition experiments.

Recently both Soames and Leigh-Clare[2] and Sherman and Stiebeling[3] have reported experiments in which they have used diets otherwise adequate but decidedly deficient in vitamin D—diets which, while less elaborately controlled, represent a sufficient approach to the ideal set by Osborne, Mendel and Park to yield results of interest. Some time ago it was noted that among the hundreds of rat families maintained at Columbia University in rooms lighted through ordinary window glass, and subsisting for generations on a mixture of wheat and milk so that whole milk powder constituted presumably the sole significant source of vitamin D, no evidence of rickets was ever observed when milk furnished about 40 per cent of the calories. When milk furnished about 25 per cent of the calories, calcium rather than vitamin D limited the calcium content of the body, for Sherman and MacLeod[4] were able to increase the calcium content of the body of these rats by adding calcium to their food, but not by adding cod liver oil.

Sherman and Stiebeling reported that when the young reared in families whose dietaries include milk to the extent of from 25 to 40 per cent of the calories are transferred at 21 or 28 days of age, soon after weaning, to a basal diet otherwise adequate but practically free from vitamin D, from 5 to 10 per cent of their calories coming from whole (summer) milk powder carried sufficient vitamin D to foster normal growth and calcification. Still smaller allowances of milk resulted in corresponding effects on calcification. Their data show further the advisability and practicability of generously endowing the young with vitamin D by regular and liberal use of milk in the family dietaries, and the lessened need for, or greater insurance resulting from, cod liver additions when the rest of the diet, including the mineral content, is well balanced and adequate, as is easily possible only when milk is abundantly supplied. At a time when our interest is focused on vitamin D concentrates, these experiments serve to reemphasize the importance of a liberal supply of mineral elements and an adequate allowance of vitamin D in the diet at every stage of life. Milk fortifies the diet at these points as well as in many other important factors.

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[1] Osborne, T. B.; Mendel, L. B., and Park, E. A.: Proc. Soc. Exper. Biol. & Med. **21**: 87, 1923; Yearbook 26, Carnegie Institution of Washington, 1926-1927, p. 355.

[2] Soames, K. M., and Leigh-Clare, J. C.: Biochem. J. **22**: 522, 1928. Leigh-Clare, J. C., and Soames, K. M.: Lancet **1**: 150 (Jan. 21) 1928.

[3] Sherman, H. C., and Stiebeling, H. K.: J. Biol. Chem. **83**: 497, 1929.

[4] Sherman, H. C., and MacLeod, F. L.: J. Biol. Chem. **64**: 429 (June) 1925.

Current Comment

MESQUITE POLLEN A CAUSE OF HAY-FEVER

Hay-fever is an allergic disorder due to the protein of various pollens Numerous tree, grass, weed and flower pollens are known to be definite causes, and it has become a matter of course to expect "crops" of sufferers during the pollinating seasons The literature is replete with articles on the various pollens that have been proved to be definite causes The layman is familiar with the fact that his hay-fever may be due to ragweed pollen while that of his neighbor may be due to the pollen of one of the various grasses Eventually it is probable that every pollen will be found to be the nemesis of some sensitive individuals In this trend it is of interest to note that the pollen of mesquite, a southwestern shrub, is coming to be recognized as a definite cause A report implicating mesquite in several cases has recently been published by Sellers[1] This is worthy of note chiefly because the probability of mesquite as a factor in hay-fever has in the past been overlooked by many investigators It is not mentioned by Balyeat and Duke in their books on hay-fever and asthma Sellers seems to be the first to report a series of cases in which it was definitely the cause Thus an apparently harmless shrub—one which has presumably been the unrecognized cause of many cases of hay-fever heretofore—bids fair to become a greater menace in the future as the population of the Southwest increases

ARTIFICIAL SPECIFIC ANTIBODIES

The dominant theory of specific antibody formation during the opening decade of the twentieth century assumed that under the stimulus of an injected antigen certain body cells undergo a functional hypertrophy, with the overproduction and internal secretion of certain hereditary specific defensive hormones Under this internal secretion theory it was hopeless to attempt the test-tube synthesis of chemically unknown specific antibodies Today the immunologists are contemplating the discarding of this historic concept and picture specific antibodies as retained biochemical derivatives of injected antigens[2] Test-tube duplication of antibody production is, therefore, within the range of logical hope Under the stimulus of this new theory, several apparently successful test-tube syntheses of specific antibodies have been reported Sdrawosmisslow and Kostromin[3] of the Bacteriologic Institute, Perm, Russia, for example, exposed diphtheria toxin to a large excess of trypsin and report that the resulting "altered trypsin" acts as a specific diphtheria antitoxin Kryshanowski[4] of the Hygienic Institute, Ufa, Russia, allowed diphtheria protoxin to act on different serum proteins, and reports that with two of these proteins the metaphorical hybrid products are specifically antitoxic Although these apparent confirmations of the newer immunology have been characterized by Kimmelstiel[5] as pseudo-antitoxic effects and not proof of specific antitoxin synthesis, the reported artificial synthesis of therapeutic antibodies is an encouraging stimulus to present methods of biochemical attack

"CONSTITUTIONAL VAGOTONIA"

It is a tempting hypothesis of clinical medicine to assume that the primary reacting tissue in anaphylaxis and related phenomena is the nervous system, and that antianaphylactic therapy should therefore be directed solely against this system Numerous attempts have been made to prove or disprove this hypothesis first by a study of anaphylactic phenomena in tissue cultures which are presumably free from nerve elements, and, secondly, by a study of isolated organs whose nerve functions have been modified or abolished by pharmacologic agents One of the latest analyses of this second type has just been published by Dr Kurt Meyer,[6] director of the bacteriologic division of the Rudolf Virchow-Krankenhaus, Berlin Dr Meyer selected, as his experimental material, isolated intestinal loops from hypersensitive guinea-pigs, which tissues he suspended in well oxygenated Tyrode's solution On the addition of specific foreign protein to the suspension fluid such loops were thrown into sharp, persistent anaphylactic contractions Isolated intestinal loops contain three nerve elements (*a*) terminals of the vagus, whose electrical stimulation causes an anaphylactic-like contraction of the intestine, (*b*) terminals of the sympathetic, whose stimulation causes intestinal relaxation, and (*c*) the intramural nerve plexus, whose sole function, so far as known, is intermuscular coordination Stimulation of the nerve plexus has never been shown to cause contraction of the intestine as a whole Dr Meyer reports that the addition of atropine to Tyrode's solution in quantities sufficient to paralyze the vagus completely does not prevent typical anaphylactic reactions Such reactions, however, are prevented by papaverine and other agents currently assumed to act directly on the muscular elements Since the known functions of the different nerve elements rule out the sympathetic and intramural systems as possible sources of prolonged contractions and since the vagus is excluded pharmacologically, he concludes that "the smooth muscle itself is hypersensitive and is thrown into contraction as a result of direct stimulation by the specific foreign protein" Unless it can be shown that there is error in this logic, that the local sympathetic and intramural nerve elements have functions not yet recognized by physiologists, clinicians are not justified in continuing such terms as "constitutional vagotonia," "parasympathicotonia," "vagotonic predisposition,'

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[1] Sellers, E. D Mesquite Tree Pollen as a Cause of Hay Fever Texas State J Med **25** 297 (Aug) 1929

[2] Manwaring W H A Critique of the Ehrlich Theory with an Outline of the Enzyme Theory of Antibody Formation The Newer Knowledge of Bacteriology and Immunology University of Chicago Press 1928 Chapter 81 p 1078

[3] Sdrawosmisslow W H and Kostromin N E Ueber die fermentative Methode der Diphtherieantitoxinerhaltung in Vitro Ztschr f Immunitatsforsch **54** 1 (Dec.) 1927

[4] Kryshanowski W N Ueber die biochemischen Beziehungen der Diphtheriebazillen zu den Eiweisskorpern des Tierorganismus Centralbl f Bakt **110**: 1, 1929

[5] Kimmelstiel O Zur Frage nach der Entstehung von Antitoxin aus Diphtherietoxin unter dem Einfluss von Trypsin, Ztschr f Immunitats forsch. **62** 245 (July) 1929

[6] Meyer Kurt Zur pharmakologischen Analyse der anaphylaktischen Reaktion Ztschr f Immunitatsforsch. u. exper Therap **62**: 270 (July) 1929

"relative tonus of sympathetic and parasympathetic," "deficiency in sympatheticotonic endocrines," or "disorganized neurocellular mechanism," in their discussion of anaphylaxis and related phenomena, except, of course, as convenient superficial metaphors to express apparent or alleged symptoms

Association News

MEETING OF COUNCIL AND COUNTY SECRETARIES OF THE MICHIGAN STATE MEDICAL SOCIETY

The January session of the Council and the Annual Conference of Secretaries of Component County Medical Societies of the Michigan State Medical Society will be held at the headquarters of the American Medical Association in Chicago, Jan 22 and 23, 1930

ANNUAL CENSUS OF HOSPITALS

The Council on Medical Education and Hospitals has just mailed out forms for its regular annual census of hospitals Information gathered in this way is published in the annual Hospital Number of The Journal, the 1930 issue of which is expected to appear some time in March That issue will contain not only newly compiled statistical data regarding the hospital field but also the revised list of institutions admitted to the Association s Hospital Register, those approved for internship and those approved for residencies in specialties

This survey furnishes the American Medical Association with the names of all physicians connected with hospitals, including staff members, resident physicians and interns, and all who use the hospital for the care of their patients Such data help the Association greatly in keeping up biographic files regarding the whereabouts, as well as hospital connections of physicians Each intern is given due credit on his permanent record in the biographic file Those who take advanced internships, or residencies in specialties, or any other work in the nature of recognized postgraduate instruction are likewise given credit.

Every physician and every hospital superintendent, therefore should see that the blank forms that have been sent to his hospital are filled out and returned.

MEDICAL BROADCAST FOR THE WEEK

The American Medical Association Morning Health Talks and Evening Health Hints from Hygeia

The American Medical Association broadcasts daily at 10 o clock, Central Standard Time, with the exception of Friday, when the health message is delivered at 9 45, over Station WBBM (770 kilocycles, or 389 4 meters)

The program for the week of November 4-9 is as follows

November 4	Convulsions in Children—What Do They Mean? by Dr John M Dodson.
November 5	Freckle Creams and Hair Removers by Dr Arthur J Cramp
November 6	What Life Insurance Means to You by Dr R. G Leland
November 7	An Afternoon with a Heart Specialist by Dr R G Leland
November 8	Keeping Out Undesirable Immigrants by Miss Mildred Whitcomb
November 9	The Family Won t Play Up by Dr R. G Leland

Evening Health Hints from Hygeia, 8 o'Clock, Central Standard Time

November 4	Feeding Tuberculosis Patients
November 5	Helping the Cripple to Help Himself
November 6	How to Make a Pretty Child.
November 7	What the Body Requires from Food
November 8	School Lunch Should Promote Health
November 9	Guard Children s Eyes During School Years

ADDITIONAL HOSPITALS APPROVED BY COUNCIL

The Council on Medical Education and Hospitals of the American Medical Association has given its approval to the following hospitals since the publication of the list in The Journal, August 31, page 715

Hospitals Approved for Intern Training

Santa Clara County Hospital San Jose Calif
West Side Hospital Chicago.
Rockford Hospital Rockford Ill
Provident Hospital and Free Dispensary, Baltimore.
Park View Hospital Rocky Mount N C

Hospitals Approved for Residencies in Specialties

Children s Hospital Denver
University Hospitals State University of Iowa Iowa City
Monson State Hospital Palmer Mass
Mount St Rose Sanatorium St. Louis
St Mary s Hospital, St. Louis
St Mary s Infirmary St. Louis
Multnomah County Hospital Portland Ore
Western Pennsylvania Hospital Pittsburgh

Medical News

(Physicians will confer a favor by sending for this department items of news of more or less general interest such as relate to society activities new hospitals education public health etc.)

CALIFORNIA

Drs Phillips and Pinkham Reelected—The California State Board of Medical Examiners, October 21, reelected Dr Percy T Phillips, Santa Cruz, as president of the board for his thirteenth term, and Dr Charles B Pinkham, San Francisco, as secretary for the seventeenth time Dr William R Molony, Los Angeles, was elected vice president

Health Officers Who Serve Without Pay—One of the most conspicuous changes in public health administration in California in the last ten years has been the merging of municipal health departments with county units (The Journal, October 12, p 1150) In spite of the mergers, it is surprising to find, the state health officer says, how many small towns there are where able physicians serve as health officers or in other public health capacities without receiving any salary whatever A large number of such men, it is said, receive from $5 to $25 a month There is one part-time health officer of a populous and prosperous community in California who serves for $25 a month. Such communities however, are becoming fewer and the tendency toward the organization of strong local health units is increasing More than half the people of California now have the privileges that come with full time health organization In some communities the position of health officer is still combined with that of chief of police or chief of the fire department or keeper of the pound or other municipal office In the extreme rural districts, it seems impossible to organize strong local public health units, and these places are where the state department of health renders a large part of its service The department considers that its chief duty is to safeguard and protect the health of communities which are unable to provide their own public health protection

Society News—Chauncey D Leake, Ph D addressed the San Francisco County Medical Society, October 8 on the pharmacology and experimental aspect of intravenous anesthesia and Dr Emile F Holman on clinical experiences with intravenous anesthesia.——The first Herzstein lectures sponsored by the University of California and Stanford University medical schools were given October 7-9, by L A. Orbelli, professor of physiology Medical Institute of Leningrad, Russia on "Recently Acquired Knowledge About the Autonomic Nervous System' These lectures were endowed by the late Dr Morris Herzstein of San Francisco——Dr Harry T Hollmann Honolulu, addressed the San Diego County Medical Society, October 8 on Molokai Leper Settlement from the Inside, and Cases of Leprosy I Have Seen, illustrated——Prof Oscar Frankl of the University of Vienna will address the eighty-first semiannual meeting of the Southern California Medical Association, November 9, Beaux Arts Hall, Los Angeles on Old and New Vienna'——Dr Clifford D Sweet Oakland addressed the Contra Costa County Medical Society, Martinez, September 10, on Nutrition of the Child'

——The Mendocino County Medical Society, Willits inspected the new Howard Memorial Hospital, September 3, at the business meeting, the president and secretary were reelected ——A new $50,000 physiologic and chemical laboratory has been completed at the Scripps Metabolic Clinic, LaJolla, of which Dr Eaton M MacKay, formerly on the staff of Stanford University Medical School, will be in charge ——The College of Medical Evangelists, Los Angeles, announces that the second annual series of medical lectures under the Colver Lectureship will be given in the evenings November 5-7, by Dr Joseph A Stucky, Lexington, Ky, formerly president of the American Academy of Ophthalmology and Otolaryngology, on (1) "Our Contemporary Ancestors (2) 'The Salvaging of These 100 Per Cent Americans', (3) 'Present Conditions and the Future Outlook."

COLORADO

Personal—Dr Clement V Marmaduke, after thirty-four years' service with the Colorado Fuel and Iron Company at Minnequa, will retire, January 1 ——Dr William C Mitchell recently went to Ann Arbor, Mich for the Colorado State Board of Health to investigate the use of the Kahn test in the diagnosis of syphilis ——Drs Walter K Reed and Homer R. Dietmeier addressed the Boulder County Medical Society, September 12, on fractures of the humerus and femur, respectively ——St. Francis Hospital, Colorado Springs, has let the contract for a $250,000 addition ——Dr Edward Jackson, Denver, was elected president of the Wills Hospital Society at a meeting, October 25 this organization is composed of ex residents and surgeons of the Wills Hospital, Philadelphia, which hospital will celebrate its centennial next year ——The Fremont County Medical Society, Florence, was addressed, October 3, by Dr Leonidas E Rupert on "Unusual Cases of Appendicitis"

DELAWARE

State Medical Meeting and Election at Farnhurst.—At the fortieth annual meeting of the Medical Society of Delaware at Farnhurst, October 8-10, the guest speakers were Drs John E. MacKenty, New York, whose subject was 'Diagnosis of Carcinoma of the Lung', Martin E. Rehfuss, Philadelphia, 'New Studies of Diseases of the Stomach' and the following, all from Philadelphia Drs Jay F Schamberg, "Treatment of Syphilis in the Light of Modern Advances", Henry R M Landis, "Reports of Rest in the Treatments of Tuberculosis", John Eiman, "Diagnosis of Malignancy', Edward A. Schumann, 'Review of Modern Views on Toxemias of Pregnancy", John A Kolmer, "Truths About Serum Diagnosis of Syphilis" and Eldridge L Eliason, "Duodenal Ulcer" Papers were presented also by many Delaware physicians There were scientific and commercial exhibits, medical motion pictures, the president's reception and dinner, and an address by Sir Frederick McGill, London, England Dr Isaac J MacCollum, Wyoming was made president for 1930, and Dr William O Lamotte, Wilmington, secretary The next annual session will be held at Dover, Oct 7-8, 1930

DISTRICT OF COLUMBIA

Dinner to Dr Sudhoff—The faculty of Georgetown University School of Medicine gave a dinner, October 19, in honor of Dr Karl Sudhoff, who recently retired as professor of the history of medicine at the University of Leipzig Among those in attendance were Drs George M Kober John A Foote, Eugene Whitmore, Prentiss Wilson, Wilfred M Barton, Joseph P Madigan, Lieut. Col Fielding H Garrison and the Rev J Coleman Nevils and Rev John Gipprich Dr Sudhoff came to this country to deliver an address at the dedication of the Institute of the History of Medicine at Johns Hopkins University, Baltimore He was a practitioner in Germany for more than thirty years, was a Prussian sanitary councilor for four years, and the first president of the German Society of the History of Medicine He is known as a translator of Arabian and Egyptian hieroglyphics and ancient Hebrew texts, and has written extensively on medical subjects relating to the Middle Ages and oriental medicine and botany

ILLINOIS

Personal—Dr Nathan J Hughes was guest of honor at a meeting of the Morgan County Medical Society in October in celebration of his fifty years of practice in the city of Waverly Dr Hughes, now about 75 years of age and still hale and hearty, graduated from the Cincinnati College of Medicine and Surgery in 1879 and after practicing for about seven years in Corinth Ky, came to Illinois, later graduating from the University of the City of New York Medical School in 1890

Society News—Dr Sidney A Portis, Chicago, addressed the McDonough County Medical Society, Macomb, October 8, on "Peptic Ulcer"——Dr Victor V Anderson, who is in charge of mental hygiene work in a large department store in New York, will be the principal speaker at the annual meeting of the Illinois Society for Mental Hygiene at the Chicago Women's Club, the evening of November 15 Reservations may be made at the society's office, 203 North Wabash Avenue, telephone, State 4477

Twenty-Five New Buildings for the Insane—The state architect announced, October 21, that contracts had been let for the construction of twenty-five fireproof buildings to supplement the state hospitals for the insane at Elgin, Lincoln and Dunning, which urgently need additional room Ten of the new buildings will be at Elgin, ten at Lincoln, and five at Dunning The architect, in designing the buildings, planned them to be a standard, so that the construction of similar buildings in the future would be as economical as possible for the state. The buildings will be E shaped with dining and recreation halls at the ends and sleeping quarters in between

Chicago

Lying-In Hospital Sold—The cornerstone of the new Chicago Lying-In Hospital and the adjoining septic pavilion on the campus of the University of Chicago will be laid, November 5, at a ceremony performed by Dr Joseph B De Lee, who has been medical director of the hospital since its foundation Others participating will be President Hutchins of the university, and Mrs Kellogg Fairbank, president of the hospital board There is said to be $1,800,000 on hand to defray the cost of building the new hospital The present Lying-In Hospital at 426 East Fifty-First Street, which has been sold, it is reported, for $750,000, will be used as the Provident Hospital on completion of the new Lying-In Hospital about January, 1931

IOWA

Hospital Beds for the Salaried Man—The Julius Rosenwald Fund, Chicago, is reported to be assisting the Graham and St. Joseph's hospitals, Keokuk, in financing the illness of the "salaried man or his family" Five two-bed rooms in each hospital have been set aside for patients who cannot pay the usual charge The Rosenwald Fund will pay 50 per cent of the difference between the charge in these cases and that for rooms which cost $21 a week

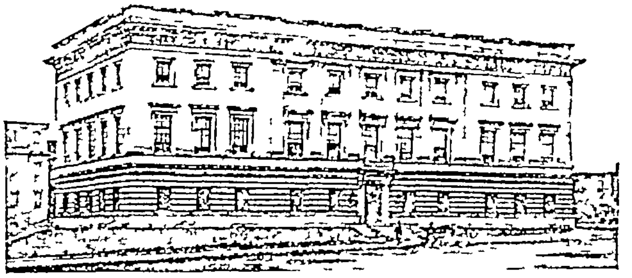
Society News—At the annual meeting of the Dubuque County Medical Society, September 10, Drs Everett D Plass, Iowa City, spoke on pernicious vomiting of pregnancy, and William Jepson, Sioux City, on surgery of the aged There was a symposium on genito-urinary calculi Dr James R Guthrie and his daughter entertained the society at a luncheon The speaker at the banquet was Judge Robert Bonson —— The Lee County Medical Society, Fort Madison, was addressed, September 12, by Drs Bruce L Gilfillan, Keokuk, on use of diathermy in treating pelvic organs, and Harold F Noble, Fort Madison, on malta fever ——Dr Anatole Kolodny, formerly of the surgical department of the state university, Iowa City, has become associated with Dr Aaron C Conaway, Marshalltown, councilor of the Fifth District Medical Society and president of the Marshall County Medical Society

MARYLAND

Dr Willis Succeeds Dr Krause—Dr Henry S K. Willis, lecturer in clinical medicine at the Johns Hopkins University School of Medicine, has been placed in charge of the Dows Tuberculosis Research Laboratory to succeed Dr Allen K. Krause, who resigned from the directorship of the laboratory to accept a position at the Desert Sanatorium, Tucson, Ariz Dr Willis graduated from Johns Hopkins Medical School in 1919, and served as assistant instructor and associate in medicine from 1919 to 1928, when he became dispensary physician of Johns Hopkins Hospital

Overcoming the State Board's Invasion—At the meeting of the Medical and Chirurgical Faculty of Maryland, October 22, Dr Alexius McGlannan, the president, urged that the membership of the society be extended in order to combat the apparent invasion of the state board of health and of the philanthropic organizations into the province of the family physician He said that the individual practitioner cannot deal with this problem The director of the state board of health, Dr Robert H Riley, who was present welcomed the appointment of a committee to act as a clearing house between the board and the *medical society* The chairman of the committee appointed to determine whether the state board is infringing on the physician's field is Dr George Milton Linthicum, Baltimore.

Dedication of the Welch Medical Library—Dr Harvey Cushing Moseley professor of surgery at Harvard University Medical School and biographer of Sir William Osler, was the principal speaker at the dedication of the Welch Medical Library of Johns Hopkins University, October 17 The new building is in Italian Renaissance, faced with variegated stone, surmounted by a heavy cornice which shadows a delicately sculptured frieze The library represents a consolidation of three great collections of books—that of the Johns Hopkins Hospital, of the School of Hygiene and Public Health, and of the School of Medicine. It has a capacity of 500,000 volumes The racks are identical with those recently installed in the Vatican Library Built around the book stacks which form the center of the building and rise to its entire height are three floors, the first two for the library and the upper for the department of the history of medicine The ground floor provides offices for the staff, bindery, smoking and coat rooms, and the kitchen From the lobby rises a marble stairway At the landing in a niche is a bust of Dr Welch, and at the top a broad corridor joining the general reading room and the great hall, designed for formal occasions In the wall is a huge fireplace At the north end is Sargent's painting "The Four Doctors" (Welch, Halsted, Kelly, Osler) The ceiling, in red and gold, bears the seals of the university, of Johns Hopkins Hospital and of the city of Baltimore and the state of Maryland With the inauguration of the Institute of the History of Medicine, the last of the units was added to Johns Hopkins University Dr William H Welch presided and declared the institute open On this occasion Dr Karl Sudhoff, professor emeritus at the University of Leipzig who came from Germany as the guest of Johns Hopkins for the purpose, and Abraham Flexner, LL D, New York delivered addresses Luncheon followed in the great hall of the Welch Library Conferences on the history of medicine were held in the afternoon The Harvey Tercentenary film, depicting the history of medicine was shown in the auditorium of the school of hygiene The dedicatory program closed with a lecture given under the Herter Foundation October 19 by Dr Otto Warburg of the Kaiser Wilhelm Institute, Berlin Professor Sudhoff is reported to have said that he had been in the large libraries of Europe and in some of the finest of Asia but had never seen such beauty combined with admirable facilities as he found in this library Dr Welch in whose honor the library is named has taught at Johns Hopkins University School of Medicine for about forty-five years as professor of pathology and as dean of the medical faculty and director of the school of hygiene and public health He is now professor of the history of medicine and director of the library



MASSACHUSETTS

Health at Somerville—Telegraphic reports to the U S Department of Commerce from sixty-two cities with a total population of about 29 000 000 for the week ending October 19 indicate that the lowest mortality rate (6 1) was for Somerville and that the mortality rate for the group of cities as a whole was 11 9 The mortality rate for Somerville for the corresponding week last year was 8 1 and for the group of cities, 12 2 The annual rate for sixty-two cities for the forty-two weeks of 1929 was 13 as against a rate of 12.8 for the corresponding weeks of 1928

Noise and Smoke Cause State Society to Move—The Massachusetts Medical Society has had its headquarters and editorial rooms at 126 Massachusetts Avenue Boston for many years Additional rooms were secured from time to time as the society grew and these quarters perhaps, would have continued to be occupied by the society for the present had it not been for the noise and smoke and other handicaps that go with such a central location The officers recognized these handicaps to editorial and committee work and frequently held conferences to decide what should be done The opinion finally was unanimous that quieter rooms should be sought So for the next two years the executive and committee rooms of the Massachusetts Medical Society and the offices of the *New England Journal of Medicine* will be at 165 Newbury Street, Boston This move in no way modifies or supplants the original expectation of accepting the offer of the Boston Medical Library The same arguments for concerted action by the society and the library still apply and the campaign for the endowment probably will soon be resumed

Medical Center to Train Family Physicians—Three old Boston medical institutions are behind the formation of the New England Medical Center which was announced October 16, namely, Tufts Medical College, the Boston Floating Hospital and the Boston Dispensary A group of new buildings will be erected consisting of a city dispensary a hospital for babies, classrooms and clinical teaching facilities and laboratory and operating equipment. The joint administrative board is composed of business men, educators and representatives of the three institutions Plans call for the enlargement of the dispensary the erection of a forty-bed land hospital of the Floating Hospital and a building to provide clinical training for students at Tufts Medical College The location will be on Bennet Street near the Park Square district, the present location of the Boston Dispensary In addition to training medical students, the center will provide graduate instruction to practicing physicians research in children s diseases, and training for about 135 nurses a year The three institutions concerned in the merger will maintain separate identities, but will share in common the facilities of the group An appeal will be made to the public for the $1 500 000 necessary for the project about $970,000 of which will be for the construction of buildings The announcement states that one principal motive behind the consolidation is to bring back to his position of importance the family practitioner and the ideals which he represents Physicians have been attracted to large centers as research men laboratory workers and teachers and fewer and fewer have gone out to the smaller communities in New England to engage in practice The old family doctor who, the statement says is the backbone of the medical profession, is dying out His revival is one of the principal objects of this amalgamation

MINNESOTA

Society News—The American College of Physicians will hold its fourteenth annual clinical session at Minneapolis February 10 14 The general chairman is Dr Solon Marx White Minneapolis and the executive secretary, E R Loveland, 133-135 South Thirty-Sixth Street, Philadelphia

Garoponlo and Von Schoppelrei.—The Minnesota State Board of Medical Examiners reports that George Garoponlo, alias Dr George, who claimed to be a doctor was fined $250, October 7, at St Paul on a charge of violating the basic science law in that he attempted to diagnose and treat an ailment which he called syphilis and which in reality was a minor skin disturbance. The fine was paid. Oscar Von Schoppelrei pleaded guilty, October 11, to the crime of abortion and was sentenced by the court to two years of hard labor in the state penitentiary at Stillwater His woman confederate was sentenced to two years in the state reformatory at Shakopee

Minnesota Alumni Meeting—For the homecoming at the Michigan Minnesota football game, Minneapolis November 16, the Minnesota Medical Alumni Association has arranged a scientific program for Friday (the previous day) which includes papers by Drs Leo G Rigler Owen H Wangensteen Jennings C Litzenberg Samuel E Sweitzer, Emil S Geist and Frank E Burch from the university Drs William R Shannon and Ernest M Hammes St Paul and George D Head Minneapolis, Edward L Tuohy, Duluth and William F Braasch Rochester Luncheon will be served at the University Hospital and there will be a dinner with entertainment in the evening The business meeting will be Saturday morning, followed by the game.

MISSOURI

Society News—The Buchanan County Medical Society, St Joseph was addressed September 18 by Dr Daniel Morton on Malta Fever ——The Jackson County Medical Society, Kansas City, was addressed September 24, by Drs Henry J Ullmann Santa Barbara Calif on "Roentgen Therapy of Mammary Carcinoma' Robert R. Newell San Francisco, on 'Roentgen and Radium Dosage Especially from a Clinical Aspect' and Charles M Richards, San Jose on 'Radiography of the Mastoid. ——Edmund V Cowdry Ph D, professor of cytology at Washington University School of Medicine St. Louis lectured on advances in the knowledge of yellow fever, October 8, under the auspices of Sigma Xi and the University of Missouri School of Medicine, Columbia ——The Jackson County Medical Society was addressed, October 22, by Dr G Wilse Robinson on "Fear, Its Effect on Behavior," and October 29, by Dr Arthur C. Clasen, and by Dr Adolph Sachs, Omaha on "Hemochromatosis." Dr Frank M Conlin Omaha, addressed the October 15 meeting of the society on "The Outlook for the Diabetic,' and Dr David S Dann on "The Uncoiled Aorta"——The Jackson County Medical Society and the state health authorities who are conducting a campaign, the Kansas City *Post* says, to stop unlicensed physicians from practicing in Kansas City, filed a charge of illegal practice against Dr Otto F Reisman, 1017½ Grand Avenue. His arrest, September 13, is said to be the first following the recent publication by the state board of health of a list of unlicensed practitioners in Kansas City (The Journal, August 31 p 700) ——The Southeast Missouri Medical Association was addressed October 1-2, at Cape Girardeau by Drs William G Patton and Millard F Arbuckle, St. Louis on 'Ear Nose and Throat Suggestions" and 'Relation of Pulmonary Infections to Upper Respiratory Infections," respectively ——Dr Leon Asher, University of Berne, lectured at St Louis University, October 17, on "The Hormonic Activity of the Liver"

NEW YORK

County Society Goes to the Fair—The exhibit of the Nassau County Medical Society at the county fair was a decided success An automatic moving picture machine displayed cartoons and facts on cancer control The object lesson on diphtheria, which was called "The House of Health," contrasted a home with children who had been immunized against diphtheria with another home in which a child had been needlessly sacrificed because it had not been immunized The society distributed literature on these subjects, and had a place for people to register questions which they wished to have answered by mail

Society News—The First District Branch of the state medical society met at Briarcliff Lodge October 25, Drs Foster Kennedy spoke on 'Neurology and the General Practitioner' C. Ward G Crampton, "The Periodic Health Examination Campaign," and John A Card, Poughkeepsie, "Medical Insurance" The president of the Medical Society of the State of New York, Dr James N Vander Veer, gave an address——The New York State Society of Industrial Medicine held its ninth annual meeting at the University of Buffalo, October 29 Among others, Drs Joshua E Sweet, New York, spoke on "Shock," and Harry E Mock, Chicago, on "Antiseptics , Claud R. G Forrester, Chicago Immobility of Fractures Associated with Mobility Treatment," and Earl D Osborne, "The Prevalence of Mycotic Dermatosis in Industry" ——A symposium on physical therapy was presented before the New Rochelle Medical Society, October 21, by Drs Charles R. Brooke, Norman E. Titus and Cassius Lopez de Victoria ——The Medical Society of the County of Nassau was addressed October 29, by Dr Luther F Warren, Brooklyn, on "Clinical Syndromes Seen in Coronary Thrombosis '

New York City

Anniversary Lecture of Academy of Medicine—The New York Academy of Medicine, Fifth Avenue and One Hundred and Third Street announces that Dr Simon Flexner, director Rockefeller Institute for Medical Research, will deliver the anniversary discourse at the eighty-third anniversary meeting, Thursday evening, November 7, on "The Infinitely Small in Biology and Medicine."

Dr Bristol Becomes Medical Director of Telephone Company—Dr Leverett D Bristol, who, for the last six years, has been director of health demonstrations for the Milbank Memorial Fund, has been appointed health director of the American Telephone and Telegraph Company with headquarters in New York. Recently, Dr Bristol made a health survey of the Bell Telephone System in the United States and Canada He was formerly commissioner of health in the state of Maine and professor of public health at the University of Minnesota

The World's Campaign Against Noise—Twenty-one civic organizations have joined with the New York *World* in an antinoise war The *World* began a series of articles, September 16, calling attention to the effects of the constant din of the city streets on health Edward K. Titus, the reporter, interviewed psychologists and health authorities to have them explain how the city's noise affected the nervous system Accompanying his articles in the *World* were news stories of arrests for violating the noise ordinances The *World* attacked principally the squawking loud speaker in front of radio shops and it quickly brought results A check-up after a few weeks showed that noise from this source had decreased about 75 per cent.

Ten Thousand Physicians in Educational Campaign—The five county medical societies of Greater New York have engaged on a campaign of public health education to be conducted by themselves to awaken the public to the value of preventive measures in maintaining health and the role of the physician as a guardian against disease During November, the importance of periodic health examinations will be emphasized. A meeting open to the public will be held at the New York Academy of Medicine, Wednesday evening November 6 The department of health is supporting the campaign and the cooperation of the public schools, and social, welfare and community organizations will be enlisted (The Journal, July 20, p 216)

Lepers in New York.—The health commissioner has revealed that there are twenty one lepers at large in the city under the supervision of the health department. According to the New York *Times* those with open sores are isolated, but the others are required merely to report regularly and are forbidden to handle food There never has been a case of leprosy in New York in which it could be shown that a person had been infected by contact with a leper in the city New York, the health commissioner says, has no need to fear the supervised lepers in its midst None of them are natives of the city There are six Russians, five West Indians, three Dutch Guianans, two Italians, and one each from the Philippines, Virgin Islands, Spain, Florida and Louisiana

Personal—The following physicians have received appointments under the Kane Fellowships in Orthopedic Surgery for periods of three years Joseph C. Risser, Halford Hallock Leonidas A. Lantzounis, Walker E. Swift Harold W Smith and Marion Beckett Howorth.——Dr James Heyman, Stockholm, Sweden, addressed the New York Obstetrical Society, October 8 on the radiologic treatment of cancer of the cervix ——Dr Walter Beran Wolfe has been appointed psychiatrist of the Children's Court of Manhattan——Dr Orrin S Wightman was host to the New York Physicians Golfing Association at the last tournament of the season at the Hudson River Country Club, September 27——A dinner was given at the New York Athletic Club, October 19, in honor of Col Albert E. Truby, second corps area surgeon at headquarters on Governors Island who has been ordered to duty in the surgeon general's office, Washington, D C——Dr Louise Pearce of the Rockefeller Institute for Medical Research, New York, has been elected an honorary member of the British Medical Society for the Study of Venereal Diseases

Society News—Dr William Allen Pusey Chicago, addressed the Medical Society of the County of New York, October 28 at the New York Academy of Medicine, Fifth Avenue and One Hundred and Third Street, on "The Corporate Practice of Medicine," and Dr Linsly R. Williams, on "The Practice of Medicine, Individual and Collective'——The Yorkville Medical Society was addressed, October 21, by Elmer V McCollum, Ph.D, Johns Hopkins University School of Hygiene and Public Health, Baltimore on "Where We Stand Now in Our Knowledge of Nutrition" Those who discussed this paper were Dr Alfred F Hess, Henry C Sherman, Ph D Dr Carl Sherwin and Walter H Eddy, Ph D——Dr Karl Sudhoff, director emeritus of the Institute of the History of Medicine, Leipzig, gave an illustrated lecture, October 22, at the Rockefeller Institute for Medical Research on 'Cos and Cnidos" under the auspices of the institute, the Charaka Club the History of Science Society, and the section of Historical and Cultural Medicine of the New York Academy of Medicine. ——The Merchants' Association announces the opening of a campaign in behalf of a proposed charter amendment which would create a new city department of sanitation

PENNSYLVANIA

Society News—At the fourteenth annual fall meeting of the Pennsylvania Radiological Society, Stroudsburg, October 25, William L Smith of the American Museum of Natural History, New York, gave a lantern slide banquet address on From the Beginning to Man", the other speakers at this all-day meeting were from various Pennsylvania cities ——The Gloucester County Medical Society visited the Jeanes Cancer and Diagnostic Hospital Philadelphia October 24 to inspect the institution and attend a special program on the early diagnosis of cancer which was given by members of the staff

Philadelphia

Hospital News—Senator-Elect Vare has purchased the spacious country estate of his brother the late Senator Vare, at Ambler, which he will give to the city as a hospital for paralytic patients The building is old English in style and is adaptable to use as a hospital

Personal —Dr Judson Daland, professor of medicine, Graduate School of Medicine of the University of Pennsylvania, has returned from a six months' tour of Rhodesia, Uganda and Tanganyika ——Dr Leopold S Vaccaro has been appointed to the surgical staff of the Philadelphia General Hospital ——Dr Russell S Boles has been appointed chief of the staff of physicians at the Philadelphia General Hospital

RHODE ISLAND

Society News —The Rhode Island Medical Society held a quarterly meeting, September 5, at the Crawford Allen Hospital, East Greenwich Drs Roland Hammond, Providence, spoke on the care of orthopedic cases, Robert M Lord, on the convalescent cardiac clinic, and Murray S Danforth, on 'Under-Water Gymnastics in Treatment of Orthopedic Diseases"

Dr Chapin Awarded Sedgwick Medal —At the annual meeting of the American Public Health Association in Minneapolis, Dr Charles V Chapin was awarded the William T Sedgwick Memorial Medal Dr Chapin, who is a past president of the association, has been health commissioner of Providence for about forty-five years (The Journal, July 13, p 127)

SOUTH CAROLINA

Personal —Dr Henry H Plowden, for several years assistant professor of pathology, Medical College of the State of South Carolina, Charleston, has been appointed director of the pathologic laboratory of the Columbia Hospital, Columbia ——Dr Washington P Timmerman is a nominee for mayor of Batesburg at the November election

Society News —Dr Charles R. May, Bennettsville, president of the South Carolina Medical Association, addressed the Seventh District Medical Association at Sumter, September 12, on "Issues Now Being Discussed Among the Profession", Drs William W Young, Atlanta "Present-Day Attitude Toward Mental Disease', Austin I Dodson, Richmond, "Some Principles in Bladder Therapy", Addison G Brenizer, Charlotte, N C, choice of operation on the stomach, and Dreyfus O Winter, Sumter, Prevention of Diphtheria", William S Burgess, Sumter, gave a report of some operative obstetric cases ——The following additions have been made to the faculty of the Medical College of South Carolina Dr Francis W Porro, lecturer in pharmacology and materia medica, Edmund J Farris, assistant in anatomy, and Joseph L Klotz, M Sc, assistant professor of pharmacy ——The Greenville County Medical Society was addressed, October 5, by Dr D Lesesne Smith, Spartanburg, on 'The Southern Pediatric Seminar", by Dr Frank H Richardson, Black Mountain, N C, on 'Postgraduate Work in Pediatrics, and by Dr William L Funkhauser, Atlanta, Ga, on Vomiting in the New-Born" ——Dr Lawrence G Clayton Central, was made president for next year of the Fourth District Medical Society of South Carolina at the October 10 meeting at Central, which was attended by about seventy-five physicians The next meeting will be at Spartanburg

TEXAS

Personal —Dr John O McReynolds, Dallas, formerly Vice President of the American Medical Association, has returned from Europe, where he was a delegate to the International Congress of Ophthalmology at Amsterdam from the Ophthalmological Association of Mexico, and from the Texas Eye, Ear Nose and Throat Society

WISCONSIN

Beaumont's Old Army Hospital to Be Restored.—The Green Bay Academy of Medicine has expressed its appreciation to Mrs Mary Woolford Hein for donating to posterity the old Fort Howard Infirmary, where years ago the late Dr William Beaumont cared for many soldiers while serving there as an army surgeon The academy appointed a committee from its members to cooperate with other committees in the community to restore this old landmark and the Brown-Kewaunee County Medical Society has voted to cooperate in this enterprise (The Journal, October 26, p 1316)

Society News —The Rock and Winnebago county medical societies had their annual golf tournament and dinner dance at the Beloit Country Club, August 21 ——The Barron-Polk-Washburn-Sawyer-Burnett County Medical Society was addressed at Barron, September 3 by Dr Harry M Coleman on "Gas Gangrene.' ——The Pierce-St. Croix Counties Medical Society celebrated at Hammond August 23 the eightieth birthday of Dr Eugene L. Boothby, who is still "hale and hearty" and in practice Drs Louis E Daugherty and Harry P Ritchie of St. Paul spoke on "Surgical Treatment of Pulmonary Tuberculosis" and "An Appreciation of the Progress of Surgery," respectively ——The Central Wisconsin Eye, Ear, Nose and Throat Society held its meeting at the Mayo Clinic, Rochester, Minn, September 27 ——The University of Wisconsin Medical Society was addressed, October 2, by Dr Leon Asher, professor of physiology, University of Berne, Switzerland, on "The Physiology of the Thyroid," and the following day by Prof F Verzar, professor of physiology, University of Debreczen, Hungary, on "Intestinal Absorption."

GENERAL

International Congress on Physical Therapy —The Fifth International Congress on Physiotherapy will be held at Liege, Belgium, Sept. 4-8, 1930, and already twenty nations have responded to the invitation. The congress will be presided over by Professors Gunzburg and De Munter, the president of the United States section will be Dr William B Snow, New York The questions proposed for consideration are (1) "Rheumatism and Physical Therapy," on which papers will be presented by Professors Gunzburg, Belgium, Van Breemen, Holland, and Wierzejewsky, Poland, and (2) "Affections of the Central Nervous System and Physical Therapy," by Dr Delherm of France. There will be sections on electrology, hydrology, radiology and actinotherapy and kinesitherapy Acceptances may be sent to Dr Dubois-Trepagne, secretary-general, 25 Louvrex Street, Liege, Belgium, with the dues of 150 Belgian francs

Society for Prevention of Blindness —The annual conference of the National Society for the Prevention of Blindness will be held at the Hotel Chase, St Louis, November 11-13, in cooperation with several Missouri boards, commissions and associations Among the speakers at the Monday luncheon will be the president of the St Louis Medical Society, Dr Cleveland H Shutt, and Dr James Stewart, the state health commissioner The Armistice Day banquet will be held jointly by the society and the Lloyd R. Boutwell Post of the American Legion The ophthalmologic section of the St Louis Medical Society has arranged a public meeting and reception for Tuesday evening, at which Dr Daniel M Velez, professor of ophthalmology at the University of Mexico, and Dr Ernst Fuchs, professor emeritus of ophthalmology at the University of Vienna, will speak. Subjects for discussion during the sessions include 'Conserving Vision in Industry, 'Trachoma," "Social Hygiene in Relation to the Prevention of Blindness," 'Vision Justice for the Young Child," and "Sight-Saving Classes"

The Most Dangerous Thing for Pedestrians —Automobile accidents in the United States last year, the National Safety Council estimates, killed 27,500 people. E. E. Free, Ph D, in the *Week's Science*, says that this represents the largest automobile death rate in any important country of the world Automobile deaths in proportion to the population have doubled since 1920, but the deaths in proportion to the number of automobiles in use have decreased about one third in the same period About six tenths of the automobile deaths in the United States, he said, are caused by an automobile striking pedestrians Less than two tenths of the deaths are due to automobiles colliding with each other A study of these accidents shows that the most dangerous thing that a pedestrian can do is to cross a street in the middle of the block, and the most dangerous thing a child can do is to play in the street. In more than nine tenths of the reported accidents, both the driver and the pedestrian were apparently in normal condition, only about 3 per cent of the drivers were intoxicated and about half as many pedestrians

Society News —At the eighteenth annual meeting in Memphis, October 7-9, of the International Association of Dairy and Milk Inspectors, Ralph E. Irwin of the Pennsylvania State Health Department was made president and Dr Paul B Brooks of the New York State Health Department, Albany, secretary Among the speakers at this meeting were Drs Brooks on "Safety First in Milk Sanitation", Alexander F Eagle on "San Francisco's Milk and Its Problems" and James A Tobey, New York, on Legal Phases of Milk Control" ——Dr William H Wilder, Chicago, was elected president of the American Academy of Ophthalmology and Otolaryngology at the annual meeting in Atlantic City, October 24 The next annual meeting will be in Chicago ——Dr George M Todd, Toledo, was made president of the New York Central Association of Surgeons at the recent annual meeting in New York, and Dr Thomas W Moorhead Terre Haute, Ind, vice president ——Surg Gen. Merritte W Ireland U S Army, Washington, D C, was installed as president of the American

College of Surgeons at the annual convention in Chicago, and Dr C Jeff Miller, New Orleans, was made president elect

Planning Negro Health Week—A conference was called in Washington, D C, October 19, by the surgeon general, U S Public Health Service, inviting representatives of health and social agencies to consider ways and means for the sixteenth annual observance of National Negro Health Week, March 30-April 5, 1930 The conference considered the results of last year's observance. Reports indicated an extension of the movement. Among the suggestions for the next National Negro Health Week were that the publications and posters be of a reduced size so that they would permit of wider distribution at a smaller expense The pictures and texts to be used were reviewed at the conference An important part of the conference was the discussion on how to make National Negro Health Week a full-time and permanent self-perpetuating movement It was voted that the National Negro Business League and the Tuskegee Negro Conference, which promote National Negro Health Week, submit for consideration a plan for the next observance, and a definite budget Roscoe C Brown, D D S, was designated to cooperate with the public health service in the publication and distribution of the official bulletin and poster R. W Emerson, representing the National Clean-Up and Paint-Up Bureau, which organization donates the four prizes given, renewed the offer of these cups and other services which might aid the promotion of this movement

Southern Medical Association Meeting—At the annual meeting of this association in Miami, Fla. November 19-22, the scientific program will be given by leaders in medicine throughout the United States, and from Canada and Cuba Tuesday will be a clinic day arranged by Miami physicians, and Wednesday a general clinic day with the program arranged by the president. The sections and other organizations will hold joint meetings, Thursday and Friday An added attraction will be joint meetings with the American Society of Tropical Medicine, the Southern Association of Anesthetists the National Malaria Committee, and the Atlantic Coast Line Surgeons Association. Scientific medicine in every phase will be covered in these sessions On Wednesday evening presidents night, Drs William Gerry Morgan, Washington, D C, President-Elect of the American Medical Association Thomas W Moore, Huntington, W Va. president of the Southern Medical Association, and Francisco M Fernandez, Havana, president of Circulo Medico de Cuba and a member of the cabinet of the president of Cuba, will speak The president s reception will be at the Coral Gables Country Club, which is said to be one of the most beautiful in the world The alumni reunions, Thursday evening, will follow the general session The men s golf tournament will be at the Miami Country Club and the ladies' tournament at the Miami Beach Municipal Golf Course A trip to Havana Cuba (not far away), has been arranged for those who desire to go after the meeting A special train will be run from Cincinnati to Miami For those who motor, Florida's roads will make the trip attractive

Change of Policy on Chautauqua Health Lectures—The Milbank Memorial Fund, New York, has published a report by the executive secretary of the American Public Health Association, Mr Homer N Calver, on the experiment in chautauqua health education which was undertaken last year under the supervision of a committee of the association The Milbank Memorial Fund financed the experiment There were four lecturers "tent-speaking for health in the first season but, in accordance with a change of policy, only one Mr Frederick M Snyder, was engaged in 1929 The report says that 375 communities in twenty three states have been reached The audiences incorporated in the chautauqua programs were estimated to total 820 000 persons Mr Calver in his report, indicates that this work has resulted in the establishment of two full-time county health units in Tennessee and many other tangible results The method was something like this An experienced health officer employed by the American Public Health Association as an advance scout visited the state and local health officials preceding the chautauqua into the towns and, in his own words unearthed the status of public health in that county and town This local health history was transmitted to the speaker before he gave his address while the advance agent whetted the interest of the townspeople in the personality and powers of the health speakers When they came to the chautauqua platform the audience was already interested in hearing what was to be said about their home town According to this report, the chautauqua provides one of the best mediums for reaching the highest concentration of intelligence in the small town.

Medical Bills in Congress—S 1837, introduced by Senator Blaine, Wisconsin proposes to amend the World War Veterans' Act to provide that veterans receiving hospital treatment under section 202 (9) of the act shall be paid a per diem allowance of $2 65, and that veterans receiving hospital treatment under section 202 (10) shall receive a similar allowance if they have dependents S 1838 introduced by Senator Blaine, Wisconsin, proposes to amend the World War Veterans Act to provide that when a veteran is shown to have a "chronic constitutional disease or a disease analogous to a constitutional disease," manifesting itself within seven years from date of discharge such disease shall be considered of service origin, and that as the list of such constitutional diseases be promulgated it shall be in force as 'from the beginning" and not from the date of promulgation S 1839, introduced by Senator Blaine Wisconsin, proposes to amend the World War Veterans' Act to provide (1) that if an ex-service man is shown to have had, within seven years after discharge, a neuropsychiatric disease and spinal meningitis, an active tuberculous disease, paralysis agitans, epidemic encephalitis or amebic dysentery developing a 10 per cent degree of disability, such diseases shall be presumed of service origin (under existing law such diseases are presumed of service origin if developing prior to Jan 1, 1925), and (2) when there is a record of "a static or chronic disease or injury made at the time of or prior to inception of active service any subsequent disability resulting therefrom shall be conclusively presumed to be connected with service. S 1913, introduced by Senator Blease, South Carolina, proposes that narcotics unlawfully possessed by a person, partnership or corporation shall be considered excisable merchandise on which the duties have not been paid or which have been brought into the United States contrary to law H. R. 4708, introduced by Representative Cochran, Missouri, proposes to authorize an appropriation of $1,500 000 to provide additional hospital facilities at Jefferson Barracks, Missouri, for disabled war veterans

FOREIGN

Personal—The king has renominated Sir George Newman as a member of the General Medical Council of England for five years from October 9——J W Bigger, professor of bacteriology and preventive medicine Trinity College, Dublin is spending October and November in the United States and Canada visiting medical schools and hospitals under the auspices of the Rockefeller Foundation.

Society for Diseases of Gastro-Intestinal Tract—The ninth congress of the Gesellschaft für Verdauungs- und Stoffwechselkrankheiten (diseases of digestive tract and disorders of metabolism) convened in Berlin Oct 16 to 18, 1929 (Langenbeck-Virchow-Haus, NW6 Luisenstrasse 58/59) The main topics on the program were critique of pharmacotherapy of the digestive tract, pylorospasm, endogenous leanness and adiposity cardiospasm, and liver disorders The chairman was *Hofrat Prof H von Haberer*, Düsseldorf Moorenstrasse 5

Tour to Egypt—The suggestion made in Vienna a year and a half ago to conduct tours to other countries to study medical conditions and methods has been successfully carried out The next journey proposed is to India by way of Egypt The party leaves Trieste, December 12, going by way of Venice and Athens to Alexandria and Cairo, where one section will go to Luxor in upper Egypt and possibly to Palestine, and another section will leave Port Said Christmas Day by way of the Suez Canal for Lahore Delhi, and Bombay to inspect hospitals and clinics Physicians from abroad wishing to join the tour should write to Aerztliche Auslands-Studienreisen, Vienna I (Austria), Biberstrasse II 1/6

Association Library Moved and Refurnished—The library and reading room of the British Medical Association London has been transferred to Hastings Hall and entirely refurnished and refitted Each reader now has a separate table There are facilities whereby members may consult books in all branches of medicine as well as books on the allied sciences The library has more than 30,000 volumes and every important medical book in the English language is added on publication The librarian and the assistant help members locate books or find references on any subject These facilities are being made use of to an *increasing extent*. The British Medical Association has now available the new annual notebook which may be had gratis on application to the secretary at headquarters Tavistock Square London W C I The new edition contains the decisions of the representative body on matters of policy a sketch of the constitution and work of the association lists of members of councils and committees and officers

Deaths in Other Countries

Theodore Tuffier of the Paris Academy of Medicine and one of the founders of *Archives medico-chirurgicales de l appareil respiratoire* October 27, following a prostatectomy, aged 71

Government Services

Army Personals

Major Charles C. Demmer has been transferred from the disciplinary barracks, Fort Leavenworth, to Baltimore for duty at the general dispensary, Major Daniel C Hutton from William Beaumont General Hospital, El Paso, Texas, to Fort Mason, California, for duty as transport surgeon, and Capt John L Meddaugh from Fort Mason to Presidio, Monterey ——Major Louis A LaGarde, Jr, will be relieved from the Presidio at Monterey about December 1 and will proceed to Fort Sill, Oklahoma, for duty, at which post Capt Alexander P Kelly will be relieved Capt Kelly will proceed to William Beaumont General Hospital, El Paso, for duty ——Capts Robert M Butler and Charles C Gans, having been examined for promotion and found physically disqualified for the duties of major by reason of disability incident to the service, their retirement from active service as major under the act of Congress, approved April 23, 1928, was announced, October 4 —— The following reserve officers have been appointed first lieutenants in the medical corps of the regular army and assigned as indicated Merritt Gartley Ringer, Chicago, to Fort Sheridan, Illinois, Robert R Estill, Bridgeport, Neb, to Fitzsimons General Hospital, Denver, Clarence Woodson Hardy, Hutchinson, Kan, to Fort Riley, Stakely Fonville Hatchette, Marion, Ala, to Fort Benning, Georgia, Charles Laurn Leedham, St George, Staten Island, N Y, to Fort Monroe, Virginia, Willis Hinton Drummond, Los Angeles, to Fort Bliss, Texas Charles Chute Gill, San Francisco, to the Presidio of San Francisco, and Marcus David Kogel, New York, to Walter Reed General Hospital, Army Medical Center, Washington, D C ——Major Gerald D France has been relieved from Fort McIntosh, Texas, and assigned to Fort Bliss, and Capt Clarence U Snider from Fort Meade, Maryland, to Fort McIntosh, Texas ——First Lieut Merritt G Ringer has been relieved from duty at Fort Sheridan, Illinois, and ordered to the William Beaumont General Hospital, El Paso for duty, Capt Gordon A Clapp from Fort Strong, Massachusetts, to Fort Ethan Allen, Vermont, for duty, and Lieut Charles L Leedham from the general dispensary, New York, to Plattsburg Barracks

Navy Personals

Rear Admiral George T Smith has been relieved of all active duty and ordered home, Lieut. Comdr John M Huff has been transferred to the Marine Recruiting Station Philadelphia, effective October 10 Lieut. Comdr James F Hooker has been transferred to the U S S *Lexington*, effective October 15 Lieut. Houston B Fite has been transferred to the Receiving Barracks Philadelphia, effective October 12, Lieut. Joseph J Kaveney to the U S S *Niagara*, Lieut Charles B Stringfellow to duty as officer in charge marine recruiting station, Charlotte, N C, Lieut Comdr Mortimer T Clement to the U S S *Oglala* Lieut. Comdr James A Halpin to the U S S *Marblehead* Lieut. Comdr Franklin F Murdock to the navy medical school Washington, D C, Lieut Comdr Russell J Trout to navy hospital Boston, Lieut. Comdr Deane H Vance to the navy air station, Hampton Roads, Va (reassignment) Lieut Comdr Paul W Wilson to the U S S *Medusa* Lieut Winfred P Dana to the navy air station, Hampton Roads, Va, for duty involving flying, Lieut Camille M Shaar to the navy hospital Pensacola Fla Lieut Roy F Cantrell to the U S S *Holland* Lieut George E Mott to the navy hospital, Portsmouth, N H, Lieut Alva A Shadday to navy hospital Great Lakes Ill, Comdr Joseph C Thompson has been relieved from all active duty and ordered home, Capt Raymond Spear has been relieved of all duty to continue treatment at the navy hospital San Diego, Calif, Lieut Paul F Dickens has been ordered from the navy medical school, Washington, D C, to the U S S *Relief*, Lieut. Frank A. Hughes from the naval academy, about November 20, to the naval station, Guantanamo Bay, Cuba, Capt John L Neilson from the navy hospital, San Diego, Calif, about November 1, to the navy hospital, Pearl Harbor

Dr Adams Becomes Medical Director of Veterans' Bureau

Dr Winthrop Adams was appointed medical director of the U S Veterans' Bureau, October 23, to succeed the late Dr Edgar O Crossman Dr Adams is a graduate of Tufts College Medical School Boston 1915 He was appointed acting assistant surgeon in the public health service in 1919, and before going to the headquarters of the veterans bureau in 1924 was medical examiner for the New England district

Foreign Letters

LONDON

(From Our Regular Correspondent)

Oct 5, 1929

The Rabbit as a Source of Postvaccinal Encephalomyelitis

In a letter to the *British Medical Journal*, Dr Myer Coplans, Col W G King and Sir William Simpson of the Ross Institute for Tropical Diseases point out that the government lymph used in eighteen out of the twenty-five cases of post vaccinal encephalitis that have occurred in this country was a product of the vaccination of calves with lymph derived from the rabbit In a recent discussion at the Royal Society of Medicine on encephalomyelitis in man and animals, Dr Tom Hare summarized the pathology of the varieties of encephalomyelitis of animals due to filtrable viruses He referred to a contagious encephalomyelitis observed in stock rabbits in 1922 by Twort and Archer, characterized by insidious onset, wasting, subnormal temperature and, in some cases, discharge from the eyes Coplans, King and Simpson therefore think that the use of a leporine virus involves a possible risk. The fact that true vaccine lymph was issued by the Army Vaccine Institute from 1880 to 1910 without disturbing accidents in two million cases shows that it is tolerated by subjects of all ages This lymph was used not only for men but also for women and children.

Climate and Health

Since the discoveries of Manson and his followers showed that the tropics might be made safe for the white man, the influence of a hot climate on the inhabitants of temperate climates has been discounted The conversion of the Panama Canal Zone from an area in which no white men could live and work into one of bustling activity illustrates the view that disease and not temperature is the enemy But, in spite of this and other examples of the benefits of tropical hygiene, a reaction is taking place Observers have recently stated that climate cannot be discounted as a factor influencing the health and work of the white races in the tropics Sir Aldo Castellani, in a review of the subject, says that permanent colonization by white men of the low-lying regions of the tropics, in which the climate is moist and hot, is not possible. Such a climate, when endured for years, diminishes resistance to disease and has a detrimental effect on the nervous system Fertility also probably decreases after the second or third generation. The climate, further, is injurious to the children, "as can easily be observed in any district of the tropics at sea level" But in Queensland white colonization seems to be successful When, twenty-five years ago, Kanaka labor was forbidden in the cane fields, it was supposed that the knell of the sugar industry had been sounded. The yield of sugar in 1905 and 1906 was 152,259 tons, in 1922, with white labor, the yield was 288 000 tons, an increase of more than 80 per cent, and white Queenslanders of the third generation work as efficiently as if they lived in temperate areas They do not show detrimental effects The death rate in Queensland in 1913 was 10 4, and the infant death rate was 63, as compared with 13 8 and 108, respectively, in Great Britain. Against this remarkable example must be set that of the white settlers in the Bahamas, which possess a subtropical climate Disease is not prevalent, but there is a large proportion of whites—descendants of the same stock that has created modern Canada Sir Aldo points out that, according to Huntington, the reason of this degeneration is the inertia produced by the enervating climate The thermometer never goes below 50 F and there is a lack of stimulus to body or mind According to Stendel, the same holds for Samoa, where the settlers of pure German stock have degenerated

Fatalities from Arsphenamine

Fatalities due to the use of arsphenamine in the treatment of syphilis are recorded less frequently than formerly. Whether this is due to an actual diminution of frequency in consequence of improved technic is difficult to say, for there has always been reason to think that such records were incomplete. However, cases do come to light from time to time, sometimes not in the form of scientific reports in medical journals but as reports of coroners' inquests. One of these was held on the body of a woman, aged 29, who died in a hospital where she had been receiving injections of an arsphenamine preparation. The government pathologist, Sir Bernard Spilsbury, gave evidence that death was due to chronic arsenical poisoning. The house physician stated that 20 000 injections were given in the hospital yearly but only one death had occurred. The deceased developed purpura hemorrhagica, which, he said, was so rare a consequence that only twelve cases were recorded. Three attempts were made to save the woman's life by blood transfusion. The coroner was satisfied that the injections of the arsphenamine preparation had been given with due care and recorded a death by misadventure.

Our Unclean Food

Dr. W. G. Savage, health officer for Somerset, in some observations on clean food issued by the Central Council for Health Education, says that evasion of the regulations intended to insure cleanliness of food is due to the general apathy of the consumer. If consumers realized the need for clean food they would get it, for the enterprising trader would promptly cater to the demand, and those who remained dirty in their practices would be left with their food on their hands, and would have to mend their ways or go out of business. We want a more active public opinion on these matters rather than more laws. If we have the laws without an active public opinion they will be largely evaded. Dr. Savage describes how traders continue to expose food to contaminations in spite of regulations.

Meat.—In the meat markets and shops, not in poor quarters only, meat is much exposed to flies and to dirt from passing vehicles and not protected by glass screens or even fly proof wire screens. Prospective buyers handle pieces of meat with none too clean fingers and so spread bacteria on them. These grow rapidly on the meat, so the initial contamination is quickly multiplied many thousandfold.

Milk.—In spite of the recent milk and dairies order, much milk is obtained from dirty cows by dirty milkers, and poured into vessels which are not clean. Examination of such milk shows huge numbers of bacteria from these sources, and this is why it sours so quickly in hot weather. Dirty handling after collection is also common enough.

Fruit and Vegetables.—Vegetables grown in manured soil are invariably contaminated at the source. Both fruit and vegetables are often exposed for sale under conditions that allow heavy pollution from dust by handling. Vegetables are washed and cooked before use, and much fruit is eaten raw.

Fish.—There is much exposure to dust and flies. If disease germs get into the interior of the fish, as they will do by growth, frying will not destroy them with any certainty and outbreaks of typhoid and other diseases have occurred from fried fish.

Bread.—The dirty handling which a good deal of bread receives is obvious, and the wrapping of bread in clean paper envelops is practiced by only a few bakers.

Theater Headaches

In a discussion on air pollution in places of entertainment Mr. W. G. Pyatt, chief sanitary inspector of Cardiff, said at the conference of the Sanitary Inspectors' Association that the chemical properties of the air in theaters and cinemas had nothing to do with feelings of discomfort. It was entirely a question of the physical properties of the air. Carbon dioxide in the air in places of entertainment never went to a dangerous point. If it grew to 1 per cent it was not dangerous. What an excess of carbon dioxide did indicate was that the air was not being changed as frequently as it should be and that ventilation was inadequate. Recently he had caused experiments to be made in Cardiff cinemas, and it was shown that a temperature of about 62 F. was the best. Leonard Hill, the president, entirely supported these views. He said that there was no chemical poison in badly ventilated buildings. The old idea that some subtle poison was exhaled from people's bodies was quite wrong. When a person felt miserable and headachy after visiting a theater or cinema it was entirely due to the fact that he had been sitting in stagnant air, which caused the want of proper loss of bodily heat, and the mucous membranes became swollen and congested. Movement of the air, not only in a direct current but in eddies, was necessary for a proper rate of cooling. The best way of making a building comfortable was to produce a proper degree of warmth in the walls. Then, if there was a current of air passing through open windows or induced by mechanical means, the atmosphere would be comfortable.

Negative Results of Ultraviolet Radiation

Some months ago the annual report of the Medical Research Council was published containing a searching criticism of ultraviolet radiation in prophylaxis and treatment (The Journal, April 20, p. 1364). The Council has now published in detail the evidence brought forward by Dr. Dora Colebrooke, who conducted an experiment with ultraviolet radiation in a Willesden (Northwest London) school. The children, divided into three groups, were kept under medical observation from August, 1927, to March, 1928. Those in one group, numbering 101, were irradiated by light from a carbon arc lamp with a high output of ultraviolet rays; ninety-four were exposed under the same conditions to the rays of a similar lamp passed through window glass, and the remaining ninety-two did not receive irradiation. The children were drawn from a crowded area and some of them came from poor homes. The conditions could not be considered so bad as to counteract any favorable effect resulting from irradiation, while they fell far short of a standard for which no added hygienic or therapeutic measures are desirable. Records were kept of height and weight, of incidence and duration of colds and allied conditions, of attack rate of infectious diseases, of occurrence of chilblains and of progress in school work. Comparison of these results shows:

(*a*) For the unexposed and unscreened lamp groups, an advantage to the former group in every respect except in progress in school work, in which the figures were equal.

(*b*) For the unexposed and screened lamp groups, an advantage to the former group in every case except in incidence of whooping cough and progress in school work, in which the advantage was to the screened lamp group.

(*c*) For screened and unscreened lamp groups, an advantage to the former group in all points except the average duration of colds, in which the figures were equal, and gain in height, in which the advantage was to the unscreened lamp group.

While accepting Dr. Colebrooke's experiment as conclusive, the Medical Research Council is careful to limit the inference that can be drawn from it. "The results simply mean that the kind of light used, applied for the periods and at the intervals chosen to children whose health is good enough for attendance at school during the months when sunlight and especially its ultraviolet component are deficient, has no measurable effect on their health." The conditions may be open to criticism. Until that has been done it is proper to doubt whether light treatment under such conditions will have any real benefit, whether the greater incidence of minor ailments among school children in an urban area during the winter months is due in any significant degree to the deficiency in the light as such. But it would be no more legitimate to generalize the application of Dr Colebrooke's negative results than it would be to suggest that because ultraviolet radiation will cure rickets it will have a beneficial effect on almost any condition of defective health.

PARIS

(From Our Regular Correspondent)

Sept 26, 1929

The Problem of Hereditary Tuberculosis

At the annual meeting of the Association française pour l'avancement des sciences, Professor Bernard delivered an address in which he discussed the present French conception of the hereditary transmission of tuberculosis On the solution of this problem depends the prophylaxis of the disease. A distinction is made between the heredity of the virus and that of the terrain The facts show that the tubercle bacillus is capable of traversing the placenta but that this is of such rare occurrence that it appears negligible in actual practice. Recent articles on the ultravirus have revealed a new mode of passing but without enlarging in reality the field of heredity The heredity of the terrain withstands no better the critical examination of the facts All the clinical, sociological and statistical arguments that have been advanced are insufficient to supply proof for the heredity of the terrain, for against it can be brought the weight of overwhelming testimony The conclusion is that while the tubercle bacillus may in rare cases be transmitted by heredity, contagion remains the essential factor in the propagation of tuberculosis and should govern the struggle against the disease.

Harmful Practices of the Natives of Cochin China

In all the French colonies, the physicians (on whom falls, to a certain extent, the task of civilizing the natives) have to combat many absurd but tenaciously held age-old practices, the persistence of which serves to perpetuate disease among the indigenous population In Dahomey, the natives have the habit, on the birth of infants, of dressing the umbilical wound with ordinary soil, which is infested with the tetanus bacillus, with the result that half of the new-born die in infancy from tetanus In Cochin China, where a peculiar conjunctivitis prevails that is as dangerous as Egyptian trachoma, the natives insist on applying to the diseased conjunctiva the flesh of frogs freshly dismembered The frogs of these regions are, however, frequently infested, in their muscles, by a minute worm that invades the intestine and often penetrates the subconjunctival tissue of human beings and causes grave damage, sometimes producing blindness Serious itching results, which induces the patient to rub his eyes constantly and thus aggravate the inflammation. Then an edema of the eyelids develops—which is frequently followed by abscesses The medicosurgical society of Saigon, Cochin China, aroused over the situation, prepared a poster in the native language and induced the governor general to display it in all the villages in order to warn the natives against this repugnant and dangerous practice.

A Peculiar Occupational Disease

Just at present, it is the open season for hunting, and consequently there is considerable consumption of game, the selling of game being authorized only during the hunting season There is therefore at present much game imported, more particularly, quail, hundreds of thousands of these birds being taken when they alight on the sandy beaches of Egypt, exhausted by their long migratory passage across the Mediterranean. They are easily captured with a net, and they are shipped in crates, chiefly to the market-halls of Paris But they are at present lean, and they are fattened rapidly in order to have them in good condition for eating To accomplish this recourse is had to the stuffing process But while that is easy to do with poultry, owing to the fact that the beak is large enough to permit a tube being slipped down the esophagus, through which food is passed by force, it is not so easy in dealing with quail, owing to the smallness of the beak. Therefore, another procedure is adopted In the cellars of the market-halls of Paris may be found some fifteen professional *gaveurs,* or bird stuffers (Italians or Poles, usually), who masticate grains and fruit and make a sort of pap of it, which is fed to the quail The operation is carried on every day for two or three weeks, each bird being considered separately and not sold until it has attained a certain weight But it has recently been discovered that this industry presents a real danger Most of these bird stuffers are from time to time pecked on the mouth by the birds, which often leads, in the course of two years, to the development of phagedenic ulcers on the lips, which degenerate into horrible mutilations, apparently the result of an aspergillosis or an actinomycosis The transmission of diseases by the beak of birds is not without precedent. A number of years ago, attention was called to a very grave type of pulmonary disorder, psittacosis, caused by small parrots of the Antilles in persons who allowed these birds to take food from their mouths, and this disorder was not only often fatal but was sometimes communicated to their entourage.

Creation of a Revolving Solarium

Heliotherapy is coming more and more into vogue. During the summer, all the beaches are crowded with persons of both sexes—who devote much patient energy to acquiring a coat of tan, though without much system and often incurring the risk of painful dermatitis But many institutes have been created in various points in France and Algeria, in which physicians administer a truly scientific heliotherapy On the initiative of Dr Saidman, a very remarkable type of solarium is being erected at Aix-les-Bains, on the borders of lac du Bourget, in Savoy A revolving solarium, turning on a pivot, is being constructed in such a manner as to receive the rays of the sun at every hour of the day, since the the front of the solarium, as it turns, continues to face the sun The solarium consists of a gallery 25 meters long, 6 meters wide, and 4 meters high. One observes in the center a projection, which is the office of the physicians and affords a view of five booths The whole structure, by means of a ball bearing mechanism mounted on rails, can be adjusted, in a general way, to the changing position of the sun and can be tipped at the most favorable angle An elevator installed in the adjacent tower (14 meters high) takes the patients up and down. In each apartment, or booth, is the concentration apparatus immense reflectors, some of them cylindric, others flat Their total surface makes possible the collection and transmission of considerable quantities of invisible ultraviolet rays, a luminous power equivalent to more than five million candles or a very appreciable caloric energy The part played by filters is particularly important, hence, they are constantly operative There are panes covered with a thin silvery film, which cuts out the infra-red light and allows the ultraviolet rays to pass, and monochromatic screens that transmit the blue, green or red light with the aid of lenses of progressive transparency But to increase the radiation tenfold and to select the rays according to the disorder to be treated was not sufficient. It was necessary, above all, to measure the dosage of the action, and that applies not only to the first clinical examination but also during the course of the irradiations Thus, the sensitiveness of each new patient is tested by a method invented by Dr Saidman, which consists in a series of successive irradiations of varying duration, the effect on the skin of these various irradiations enables the operator to choose the best irradiation for the patient in question. But that evidence has not been considered sufficient, hence, to the irradiated body surface of each patient a thermopile, which transmutes solar rays into a measurable current, is applied. All these indicators will be connected with registering apparatus located in the central office of the therapeutist. If the quantity of energy being received exceeds the prescribed dose, the graph will indicate the fact by abnormal points, and the physician will reduce the radiation by manipulating the reflectors or the lenses Finally, a spectograph of high power will show constantly the variations of luminosity, thus enabling the physician to shorten or to prolong the sitting in accordance with the caprices of the weather Everything has therefore been provided for—even the momentary eclipse of the sun by a passing cloud, for, if the diminution of the natural solar radiation becomes too marked, a large number of artificial "suns' are immediately lighted ultraviolet or infra-red lamps, which prevent any possible interruption in the flow of radiant energy

ITALY

(From Our Regular Correspondent)

Sept 15, 1929

The Honor Roll of the Victims of Roentgen Rays

Professor Mariani, chief surgeon of the Ospedale di Schio has begun the preparation of an honor roll containing the names of Italian physicians who, in the exercise of their profession, have been seriously injured by the action of roentgen rays or radium. There are many victims of duty who at present are little known in Italy The most frequent cases date back to the earlier years of the use of radiology, but in recent years, in spite of the fact that the means of protection have been perfected, the number of injuries to physicians has not been slight, owing to the increasing use of roentgen and radium radiations The president of the Societa di radiologia has approved the plan.

The Death of Professor Francioni

The death, at Bologna, of Prof Carlo Francioni, director of the university pediatric clinic, has been announced Professor Francioni was born in Florence, in 1877 He was graduated in 1901, and entered the pediatric school at Mya, in which he became "*assistente*" and later '*aiuto*' (aid) In 1915, he was appointed to the chair of pediatrics in Bologna, which he held until his death. He is the author of fifty-two published works, in addition to collaborative articles in a number of treatises Of chief importance are his studies designed to explain the mechanism of the action of serotherapy and questions of like nature The results of his researches are contained in fourteen printed articles He was one of the first advocates of antimeningococcic vaccinotherapy, at a time when every one was opposed to serotherapy He was the first to propose intraspinal serotherapy in diphtheria He invented an apparatus for the measurement of intraspinal pressure in nervous diseases in children. As the result of his researches on convulsions in the first months of infancy, he developed the idea of a pathologic congenital spasmophilia, in which the predisposing constitutional factor is very important. In his most recent works (1926-1928) he referred the syndrome of acrodynia to an encephalitic lesion localized in the diencephalic and the mesencephalic centers His conception, which was based solely on the clinical finding, was confirmed later by pathologic anatomy Francioni was a confirmed "constitutionalist" and emphasized the importance of early researches on the nursling with regard to the morphologic types of Viola, in order to recognize and follow the particular evolution of the individual He bequeathed all his property to the Clinica Pediatrica of Bologna

The Riberi Prize Contest

The Accademia di medicina of Turin has opened the contest for the fifteenth Riberi prize of 20,000 lire ($1,050) Scientific works on any subject pertaining to medical disciplines provided they constitute real progress in the field with which they deal may be entered. Printed or typewritten works (two copies being required) composed in Italian, Latin, French, English or German are acceptable. Printed works must have been published since 1927 In order to be given consideration, books to be entered in the contest must be received by Dec. 31, 1934

Aid for Mental Patients in the Province of Milan

At the Mombello psychiatric hospital in Milan, the newly chosen administrators of the province of Milan met recently to study the problem of how best to care for the large number of mental patients From reports presented at this meeting it became evident that the total number of mental patients in the province receiving aid at the present time amounted to about 4 500, 3,400 of whom are cared for in the psychopathic hospital at Mombello, where a suitable work colony has been created The remainder are found in institutes for mild cases, such as the Ospedale di S Colombano, which cares for 740 chronic patients To provide for future accretions, it was decided by a committee presided over by Professor Antonini not to construct new buildings, by way of enlarging the Ospedale di Mombello, but rather to develop further the institutions for mild cases, with dispensaries and ambulatoriums (three at Milan and four out in the province), and a retreat for cases of chronic incapacity In addition, a workshop (*casa di lavoro*) for mild chronic cases and agricultural colonies for discharged patients will be established Within two years, however, it is planned to erect a provincial psychiatric hospital and also a psychiatric clinic at the University of Milan. For the criminal insane the commission proposed the creation of '*frenocomi statali di sicurezza*"

A New Otorhinolaryngologic Society

The Italian charter members of the recently established international society Societa otorinolaringologica latina are Professors Bilancioni, Brunetti, Calamida, Caliceti, Citelli and Milan.

The society will hold a congress every year, at which will be discussed *only* the official topics and general scientific question. Individual communications will not be accepted. The first congress will be held in Madrid Papers will be presented on these two topics (1) septicemia of otitic origin, and (2) sinus phlebitis The question for general discussion will be "The mechanism and the reactions of labyrinthine stimulation."

Meeting of the Medicosurgical Academy

The recent meeting of the Accademia medico-chirurgica del Piceno was devoted almost exclusively to a consideration of the pathology of the gallbladder Professor Baccarani discussed the chronic gallbladder syndromes not due to calculi He explained the etiology and pathogenesis of these pathologic forms, which are encountered rather frequently in general practice and pointed out that, in addition to the inflammation that is characteristic of the gallbladder, one may note often a causal influence of a hypotonic or atonic state, or of an abnormal position or a peculiar anatomic state. He emphasized the diagnostic importance of biliary drainage and interpreted the results He elucidated some of the types of chronic cholecystitis, among others, the pseudotuberculous type and the cachectic type, also the cases of repeated attacks of pain in the region of the gallbladder due to numerous varieties of gastro-intestinal syndromes of a dyspeptic nature and, in some cases, to the influence of a right pneumothorax. The treatment will not be the same for all forms of chronic cholecystitis not due to calculi but will vary according to the pathogenesis of each case He has found very efficacious medical biliary drainage with the aid of the Einhorn sound, the technic of which he described

Professor Cappelli considered the indications for operation in cholecystitis The speaker advised a careful selection of cases that prove to be incurable medically, for his long experience leads him to regard the operative risks including angiocholitis, as far from inconsiderable In cases of profound changes of the walls of the gallbladder he accepts the indications for cholecystectomy but recommends returning to the conservative, operation of Loreta, cholecystendysis, whenever the walls themselves are practically normal Professor Montanari discussed also cholecystography, which is based on the elimination of tetrachlorphenolphthalein through the biliary tracts The administration may be by mouth or the venous route Rapid evacuation of the gallbladder is then effected by the Bronner method Montanari presented also a number of radiographic projections of normal and pathologic gallbladders, which aided his elucidations

Professor Caucci reported the results he had secured in his personal cases In fifty interventions on the gallbladder he encountered forty-three true cases of cholecystitis, there were two deaths The other cases were associated with appendicitis, duodenal ulcer, pancreatitis, and the like

Professor Sala spoke on the subject of hepatoligamentopexy as treatment for stenosis of the biliary tracts in hepatogastroduodenal ptosis The speaker employed this method up to 1922 and effected complete cures in seven cases In twelve other cases he combined hepatopexy with cholecystectomy, with the best results These cases were supported by the roentgenologic observations of Dr Marzi

On the basis of thirty-five personal cases in which he operated, Professor Sbrozzi dealt with cholecysto-appendicitis It is a frequent type, and occurs chiefly in younger persons, and more particularly in the female sex. Medical treatment may be tried, if it fails, surgical treatment is indicated The preferable operation (to be carried out during a remission) is removal of both gallbladder and appendix

Professor Torrini reported on eighteen interventions that he performed in cholecystitis

Professors Santini, Sette and Frattini took part in the discussions

JAPAN

(From Our Regular Correspondent)

Oct 1, 1929

Epidemic of Cholera

A Chinese member of the crew of a Norwegian steamer was found to have cholera in the Yokohama quarantine station, July 7 It was at the time when the metropolitan police were planning to take precautions against the epidemic in China, where more than 200 new cases daily were being reported in Shanghai A passenger on the *Taireimaru* which had touched at Shanghai, was found to have cholera at Tairen, South Manchuria, July 9 The authorities there disinfected the steamer but not before more than 400 passengers had dispersed A fireman of the *Texus* was found to have cholera in Nagasaki, July 12 and all seabathing places were closed A freight handler on a canal boat in Osaka died of a sudden illness, July 16, which was probably cholera. The Osaka authorities prepared 25,000 tubes of vaccine for prophylaxis against the disease The health bureau in Tokyo took precautions against its invasion into the capital

A freight handler's young son in Tokyo fell ill at 3 and died at 5 in the afternoon July 21 On the same day, the wife of the captain of a sailing ship died of the disease in Kobe. Bathing in the sea and harbor and in the neighboring places was strictly forbidden thereafter A freight handler's wife was reported ill with cholera in Osaka, July 23, and more than 2,000 freight handlers and sailors were vaccinated by the health officers A fifth case was found in the hospital of the Osaka Medical College and a sixth case in a washerman living on the Yodogawa River, July 24 A patient with the disease died in the hospital of the Kyoto Imperial University on the same day Three more cases were reported in Osaka, July 25, two of the patients were the wives of freight handlers A new case was reported in Shimonoseki, July 27 The man had been ill since the 26th, and died on the evening of the 27th. Two more cases in Osaka and one case in Kobe were reported the same day Five more cases were reported in Osaka July 28 The health bureau gave lectures on the precautions to be taken against the disease to seventy fishermen in the Commerce and Industry Hall of Marunouchi, Tokyo Three more cases were found in Osaka, July 29, in spite of all the preventive work done there by the authorities and the people Two more cases were reported in Osaka, July 30 One of the patients was a merchant in a market place. The market place was disinfected and closed temporarily A new case was found in Yokohama, July 31 Four new cases were reported in Osaka, September 3 Two babies died of a suspicious disease at Sakai, a city near Osaka, on the same day Three new cases in Osaka and a new case in Hiroshima were reported, September 4 The thirty-sixth case in Osaka was reported September 5 A tragic story was told concerning a family entirely wiped out by the epidemic there. A new case was also discovered in Tottri, a city near Osaka

The epidemic seems to have been checked by the sudden cool weather caused by the rainy week beginning September 10 A new case was reported, September 20, in Aomori, in the northeastern part of the mainland It is believed that the disease was brought by a freight steamer from the South Sea Islands The home ministry published a statement on the total number of cases (109) in Japan on September 10

Studies on Mountain Sickness

Mountain sickness on Mount Fuji has been studied by the professors of the Keio University for the last ten years Drs T Kototo, T Iwawo and other medical scholars who are interested in mountaineering studied the disorder among the mountaineers on Mount Hakuba last summer The results will be published in the near future

The Nightingale Medal to Japanese Nurses

Two Japanese nurses are to be awarded the Florence Nightingale Medal at the thirteenth international Red Cross conference They are Miss Midori Kagawa, chief nurse in the Red Cross Hospital of Tokyo, and Miss Kei Mizuno, chief nurse in the Gumma Branch of the Japan Red Cross Society Only two other nurses have been awarded the medal in Japan.

Noguchi Medal

The French government has awarded a gold medal in honor of the researches made on infectious diseases by the late Dr Hideyo Noguchi, who died in Africa during his studies on yellow fever The medal was given to his nephew from his native place Aizu, Fukushima Prefecture, August 26, through the department of foreign affairs Professor Stein of Ohio University arrived here, September 3, in order to collect material for the preparation of Dr Noguchi's biography He went to Noguchi's birthplace to stay for about two weeks

Encephalitis

An epidemic of encephalitis prevailed in this country in 1925 Only a few cases have been reported since. Last summer, however, during the turmoil caused by cholera, fifteen new cases were reported in Ehime Prefecture, and three of them ended fatally, September 24 In Kobe twenty-four cases were reported September 28 Sixty seven of 151 cases, including nineteen new cases, have been reported as ending fatally Four of eight patients died in the city of Fukuyama and the soldiers of the Fukuyama regiment had to be quarantined in their barracks for a time Fourteen cases have been reported from Kurashiki In Osaka, the number of the cases has amounted to twenty-one including four new cases Okayama is reported to have had 213 cases with eighty deaths, while Kagawa has had 180 cases with seventy-two deaths Both prefectures were in the districts in which the epidemic prevailed in 1925 Ehime has had ninety cases with five deaths Other districts in which the epidemic has prevailed are Tokyo, seven cases, five of

In microscopic sections the pituitary of the thyroidectomized rat and the rat with combined thyroidectomy and adrenalectomy have identical changes; namely, granulated acidophil cells have disappeared entirely in both. Each pituitary is filled with large basophilic thyroidectomy cells, many of which contain intracellular hyaline material. These thyroidectomy cells are regarded as cells producing TSH which is secreted in excess when the pituitary is released from the inhibiting effect of thyroxin.

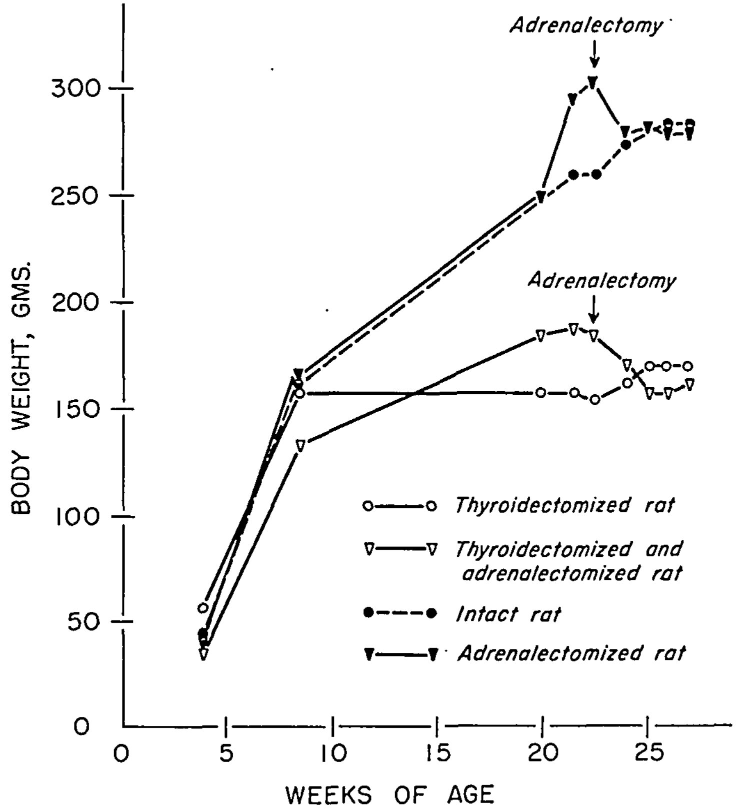


FIG. 3. Growth curve of litter-mate rats (49R) that are illustrated in Figures 2 and 3.

DISCUSSION

It is of interest to attempt a correlation of the structural with the functional changes in the pituitary. Several questions arise: Is the disappearance of granulated acidophils in the pituitary of the thyroidectomized animals a manifestation of decreased secretion of STH ? Does this mean that STH secretion is influenced by thyroid activity? Are the numerous acidophils in the pituitary of the adrenalectomized rats responsible for accelerated hair growth? Unfortunately, a simple explanation that body growth and hair growth are both manifestations of STH and are both correlated with numbers of acidophils does not hold because we find that accelerated hair growth occurs in rats with combined thyroidectomy and adrenalectomy in which no granulated acidophils are present in the pituitary.

In spite of this apparent inconsistency, there is evidence accumulating from biochemical studies that growth hormone secretion is altered after thyroidectomy and after adrenalectomy.

That there may be an increased amount of growth hormone in adrenalectomized animals is indicated by the experiments of Engel (1951b) who states that in the adrenalectomized animal there is an increased rate of protein synthesis and a decreased rate of protein breakdown and thus presumably the adrenalectomized animal should show a greater growth rate than normal, if it were not obscured by reduced appetite of the animal. Analyses of the carcasses of normal rats restricted to the same food intake as adrenalectomized animals suggested chemical growth in the latter. He cites a thesis by J. Winternitz (1942) indicating that specific tissues may show enhanced growth rates after adrenalectomy.

Tepperman, Engel, and Long (1943) also cite Winternitz' experiments (1942) as suggesting that the removal of the adrenals which normally promote protein catabolism results in a relative hyperactivity of the anterior pituitary in promoting anabolism.

Wyman and Tum Sudan (1943) found increased growth of the epiphyseal cartilage of adrenalectomized rats, interpreted to mean that adrenalectomy releases growth hormone from the pituitary. It is of interest that they found that this increased growth of cartilage began near the end of the 2nd week after adrenalectomy, i.e. exactly at the time that elapsed before hair growth was accelerated in the present experiments.

Other biochemical studies seem to have a bearing on the question of whether growth hormone production is altered after thyroidectomy and after adrenalectomy. Hoberman (1950) in studies of amino acid and protein metabolism gives data which suggest the possibility that the secretion or synthesis of growth hormone is influenced by the activity of the thyroid gland, and he suggests that "in hypothyroidism the rapidity of breakdown of amino acids is due to a release of the catabolizing system from the inhibiting influence of growth hormone." He states that "adrenalectomy appears to be responsible for stimulation of protein synthesizing reactions whether performed on normal or hypothyroid rats" and he finds that "the nitrogen output is significantly lower in the absence of both adrenal and thyroid glands than after extirpation of either gland alone."

Is hair growth an expression of growth hormone (STH) activity? Hypophysectomy causes great retardation in regrowth of hair (Snow & Whitehead, 1935). We should not expect hair growth to be completely abolished by hypophysectomy, because, as Engel (1951a) suggests, it is generally recognized that "hormones do not initiate or abolish any specific metabolic reaction, but simply modify the rate of existing ones." Both hair growth and body growth are suppressed by administration of ACTH or cortisone to the normal animal, so one would expect the growth of both would be increased after adrenalectomy, if the effects of growth were not masked by decreased food intake.

Van Dyke *et al.* (1950) suggest that "production of growth hormone by the pituitary is not under the control of the blood level of growth hormone." The evidence summarized in the present report suggests the possibility that the level of adrenal cortical hormone in the blood may be a means of controlling the rate of growth hormone secretion. Thus removal of the adrenals would remove an inhibition to the pituitary and release growth hormone secretion.

In the homeostatic regulation of carbohydrate metabolism, it is well established that the hypoglycemic effect of insulin secretion by the pancreas is counterbalanced by the 2 opposing factors of (1) anterior pituitary diabetogenic factor and (2) the adrenal cortical factor promoting gluconeogenesis. If the adrenal cortical factor is removed by adrenalectomy, one might anticipate that the anterior pituitary might compensate by secreting extra diabetogenic factor. Consistent with this idea is the fact that adrenalectomized rats, if given sufficient NaCl, will tend to maintain adequate levels of blood sugar. Since the anterior pituitary diabetogenic factor has been attributed to growth hormone STH, one might postulate that after adrenalectomy there might be compensatory increased secretion of growth hormone.

We should not expect accelerated hair growth to be demonstrable in such animals as dogs and cats in which fatal adrenal insufficiency occurs too rapidly.

Aside from these theoretical considerations, the observation of hair regrowth after shaving offers a very simple method for estimating the adequacy of thyroidectomy or adrenalectomy during the course of long time experiments, and may also give an estimate of rate of STH secretion even when body weight can not be taken as an index, due to inadequate food intake.

The lack of correlation between granulated acidophils and apparent STH secretion can not be explained at present. The absence of acidophil granules does not necessarily mean that no STH secretion is produced. The actively secreting cell discharges granules and then the cell reverts to the chromophobe appearance. Lack of granules may mean that there has been an accelerated rate of secretion by the acidophils to the point of exhaustion, where all hormone is discharged into the circulation and none is stored in the cell. Secretion rate and storage need not run parallel.

Whatever is the cause of the accelerated hair growth after adrenalectomy it is obvious that the changed metabolism takes several weeks to develop. This change, once established, is maintained for long periods. In the present experiment, no point was reached at which the acceleration of growth of hair disappeared.

Ralli and Graef (1945) reported that DOCA prevented the stimulating effect of adrenalectomy on regrowth of hair. At present it does not seem possible to place this finding in relation to the results of the present experiments.

A study of hair growth after combined thyroidectomy and adrenalectomy is recorded by Butcher (1941) who devised experiments to find out if accelerated hair growth after adrenalectomy was accomplished by thyroid secretion. He used very young rats, underfed so as to reduce the rate of hair growth, and thyroidectomized at 36 days of age. In some rats adrenalectomy was done at the same time, in others adrenalectomy was done 7–10 days and 3 weeks after thyroidectomy. He concluded that thyroidectomy had no effects on hair growth after adrenalectomy. However, his experiments do not extend over a sufficiently long period to give time for adequate decay of thyroid hormone in the circulation, nor could advanced pituitary changes take place. Furthermore, the periods of hair growth he studied were only 8 or 9 days. In brief, in Butcher's experiments, conditions were very different from those in the present experiments.

SUMMARY

Regrowth of hair after shaving was studied in groups of rats of the same sex and age: (1) rats thyroidectomized at 29 to 109 days of age; (2) rats thyroidectomized at the same time as in the preceding group and also adrenalectomized 34 to 163 days after thyroidectomy; (3) rats adrenalectomized at the same time as in preceding group; (4) intact controls.

Confirming previous studies, it was found that thyroidectomy retarded regrowth of hair.

In adrenalectomized rats, with their thyroids intact, there was a latent period of 12 to 23 days, after which hair grew diffusely instead of in the normal cycles, and at an accelerated rate. After adrenalectomy in previously thyroidectomized rats, there was a longer latent period of at least one week more than in those with thyroids intact, followed by a diffuse regrowth of hair that eventually equalled the growth of hair in adrenalectomized rats with thyroids intact. Microscopic appearance of skin was consistent with the gross findings.

After thyroidectomy, pituitaries showed the usual disappearance of acidophils and development of basophilic "thyroidectomy" cells. Subsequent adrenalectomy did not reverse these histological changes; that is, acido-

philic cells did not reappear. Nevertheless, from the results of other investigators, it seems likely that adrenalectomy does cause the pituitary to form more growth hormone.

In view of the fact that STH is commonly ascribed to the acidophil cells of the pituitary, the absence of acidophils in rats with combined thyroidectomy and adrenalectomy, in which accelerated regrowth of hair occurred, is an apparent inconsistency for which there is no explanation at present.

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A RAPID METHOD FOR THE QUALITATIVE AND QUANTITATIVE ESTIMATION OF THE PHYSIOLOGICAL ACTIVITY OF THE ADRENAL CORTEX[1]

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IT IS apparent to investigators interested in the physiology, pharmacology, or physiological chemistry of the adrenal cortical hormones that a rapid method for the qualitative and quantitative analysis of the hormonal secretions of the adrenal cortex would be of considerable value. It is the purpose of this communication to describe such a procedure and to present preliminary data resulting from the application of these techniques.

EXPERIMENTAL

For this particular study, dogs were chosen for the experimental animal. The dogs were sacrificed by an intracardiac injection of a lethal dose of nembutal. The adrenal glands were quickly removed and placed in ice. After removing the connective tissue and fat, the tissues were blotted, weighed, and slices prepared with a Stadie slicer. An aliquot was reserved for nitrogen analysis. The remainder of the slices were incubated in a large Warburg flask containing 20 ml. of the animal's own serum along with 10 mg. of adrenal cortical stimulating hormone (Armour lot #128-105R, potency 1.6 times that of LA-1-A standard), 50,000 units of penicillin, and 0.1 gm. of streptomycin. The vessel was gassed with 95% O_2 – 5% CO_2; the bath temperature was 37.5° C. and the time of incubation was 20 hours.

Following incubation, the adrenal slices and incubating medium were homogenized and subjected to fractionation similar to that described by Tennent, Whitla, and Florey (1951). The aqueous homogenate was treated with 5 volumes of acetone and the precipitated proteins filtered off. After removal of the acetone by vacuum distillation, the residual aqueous phase was extracted 5 times with ethyl acetate and dried over sodium sulfate. The bath temperature for all distillations was 40° C. The ethyl acetate was

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DISCUSSION

It is clear that following the incubation of relatively small amounts of adrenal cortical tissue, significant quantities of cortical steroid hormones can be recovered by the partition and chromatographic techniques. It is quite evident that the incubating procedure is of importance in estimating the actual elaboration of steroid hormones. The largest quantity of the predominating steroids obtained by direct isolation employing similar chromatographic techniques from beef adrenal glands was 400 μg. per pound of Compounds B and A (Zaffaroni and Burton, 1951). In the present experiments, as much as 100 μg. of Compound F could be recovered after incubating 1 g. of adrenal tissue. In the studies of beef adrenal glands cited (Zaffaroni and Burton, 1951), Compound F was present in the gland in quantities considerably less than Compounds B and A. In the present experiments with dog adrenal slices, Compounds F and E were predominant. It is certainly possible that this may be due to the difference in the species of animals employed (cf. Pfiffner, 1942). The discrepancy between the quantity of hormone present in the gland and the actual physiological elaboration receives support from the experiments of Hechter *et al.* (1951) who found that the chief hormones elaborated by perfused beef adrenal glands were Compounds F and B.

It is hoped that these techniques will be useful in evaluating adrenal cortical function during various stressing conditions. Some experiments along these lines are in progress and will be reported later.

SUMMARY

A relatively rapid method for the qualitative and quantitative measurement of the steroid hormone production of adrenal cortical tissue has been described.

The following steroids have been identified and quantitative estimation of their elaboration determined: Compounds F, E, A, S, and B. Compound F is produced in the greatest quantity; Compound E somewhat less, and smaller amounts of A, S, and B were found.

It is apparent that the steroid hormones recovered after incubation differ greatly in a quantitative manner from those recovered by direct isolation from the adrenal cortex.

ACKNOWLEDGEMENTS

The author is indebted to Dr. Karl Folkers of Merck and Co. for the samples of Compounds E and F, to Dr. William J. Haines of the Upjohn Co. for Compound B, to Dr. E. M. Richardson of the University of Pennsylvania for Compound A and desoxycorticosterone, to Dr. Don H. Nelson of the University of Utah for Compound S, and Dr. Irby Bunding of the Armour Co. for the ACTH.

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A QUANTITATIVE METHOD OF DETERMINATION OF THE DIABETOGENIC ACTIVITY OF GROWTH HORMONE PREPARATIONS*

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A NEW, rapid and quantitative method of determination of the diabetogenic activity of growth hormone preparations has been developed, based on the effect of this hormone on obese-hyperglycemic mice. The metabolic characteristics of this new Mendelian recessive condition have been described in previous publications (Bleisch, Mayer and Dickie, 1952; Guggenheim and Mayer, 1952; Mayer, Russell, Bates and Dickie, 1952; Mayer, Russell, Bates, and Dickie, 1952).

Particularly relevant to the question discussed here are the characteristics of the hyperglycemia in the obese mice. This develops as a progressive condition, usually after the 12th and before the 18th week of life. The precise time of onset seems to be conditioned by the presence or absence of genes other than "obese" in the genetic composition of the obese mice. The hyperglycemia is sensitive to the amount and the nature of the diet. It is highly sensitive to growth hormone, being either precipitated (during the prediabetic diet) or very much exaggerated (during the diabetic or hyperglycemic phase) by a single injection of growth hormone. By contrast, the blood sugar of non-obese mice are unaffected even by prolonged, massive treatment. Decreased glucose phosphorylation rates have been implicated in the etiology of the hyperphagia, in the light of the glucostatic theory of regulation of food intake (Mayer, 1952; Mayer and Bates, 1952).

Recent results (Mayer, unpublished) indicate that the obese animals are characterized by hyperfunction of the alpha cells of the pancreas and overproduction of a pancreatic hormone other than insulin. Growth hormone appears to be the trophic hormone corresponding to this new pancreatic factor.

METHOD AND RESULTS

The property of single injections or of a few injections of growth hormone to increase drastically the blood glucose of obese-hyperglycemic animals is the basis of the proposed

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method. Although reliable results were obtained with single injections in diabetic animals, maximum precision and homogeneity was obtained when using obese mice still in the prediabetic period injected for 3 consecutive days with 3 equal doses and effecting the determination on the 4th day. The Folin Ferricyanide colorimetric method (Folin, 1930, 1932) following the Somogyi method of precipitation (Somogyi, 1945) was used. The animals were 3 to 4 months old, weighed 40 to 50 g. and were fed Purina Laboratory Chow. Sex introduces no variable in the determination.

TABLE 1. EFFECT OF THREE CONSECUTIVE DAILY INJECTIONS OF GROWTH HORMONE ON THE BLOOD SUGAR OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Dose (mg. growth hormone)	none (saline)	0.1	0.2	0.5	1	2	4	10
Number of animals	10	8	8	7	5	4	4	4
Mean blood sugar (mg. per cent)	160	207	218	230	248	303	355	507
Standard deviation (mg. per cent)	±20	±12	±9	±6	±3	±4	±15	±32

A standard curve (given in tabular form in Table 1) was established using a usual (Armour) preparation. Doses given are the daily dosage. The results were shown to be independent of the method of preparation by comparing the standard Armour hormone with a preparation made according to the directions of Raben and Westermeyer (1951, 1952) for the isolation of "non-diabetogenic" growth hormone. (Comparison was made on the basis of equal nitrogen retaining activity or growth promotion in the hypophysectomized rat.) Possible errors in the method due to contamination by other pituitary hormones were studied by determining the effect of adding daily large supplements of ACTH and thyrotrophic hormone to the standard growth hormone preparation—ACTH (Armour) even in large amounts did not modify the results. Thyrotrophic hormone (Armour) increased blood sugar values only if doses of the same order of magnitude than growth hormone were used. The preparation used (Armour) did, however, show some growth activity. (Thyroxine, in doses up to 100 μg. per animal does not increase blood sugar levels (Mayer *et al.*, in press).) These results are summarized in Table 2.

TABLE 2. EFFECT OF MODE OF PREPARATION AND OF ADDITION OF OTHER PITUITARY HORMONES ON THE BLOOD GLUCOSE RESPONSE OF YOUNG HEREDITARILY OBESE-HYPERGLYCEMIC MICE

Treatment	Number of animals	Mean blood glucose and standard deviation (mg. per cent)
None (saline)	10	160 ±20
0.2 mg. standard Armour growth hormone	8	230 ± 6
0.2 mg. Raben et al. growth hormone	6	225 ±27
0.2 mg. growth hormone + 0.2 mg. ACTH	8	220 ± 9.2
0.2 mg. growth hormone + 0.2 mg. thyrotropic hormone	6	235 ±15

DISCUSSION

This method is simple, quantitative and speedy. It has been repeatedly used in this laboratory in studies of purification of growth hormone. It may be emphasized again that, while the experimental conditions described represent ideal circumstances for the carrying out of the determination, it is possible, whenever available material is limited, to carry out the determination using single doses. A standard curve should be established with single doses of the reference preparation. Sensitivity, though not reproducibility, is increased when more diabetic mice (older animals) are used and when a high glucose diet is fed to the animals.

Preliminary results, indicating many similarities between goldthioglucose obesity (Waxler and Brecher, 1950) and the hereditary obese hyperglycemic syndrome suggest that goldthioglucose-treated animals, more readily available, may be used in this bioassay.

SUMMARY

A new method for the determination of the diabetogenic activity of growth hormone preparations, making use of the trophic effect of growth hormone on the blood glucose of hereditary obese-hyperglycemic mice is presented. The method is rapid and can be used with hormones prepared by different methods, even in the presence of other pituitary contaminants.

Acknowledgement

As in previous studies, the mice used in the experiments described here were obtained through the generosity of Miss Margaret M. Dickie of the Jackson Memorial Laboratory, Bar Harbor, Maine.

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HORMONAL FACTORS INFLUENCING FAT DEPOSITION IN THE INTERSCAPULAR BROWN ADIPOSE TISSUE OF THE WHITE RAT[1]

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INTRODUCTION

IT HAS already been reported that prolonged exposure to cold leads to a hypertrophy of the interscapular brown adipose tissue in the rat (Pagé and Babineau, 1950). Since in such animals metabolism is necessarily enhanced, it was thought worthwhile to investigate the effects of hyperthyroidism on the brown fat of rats kept at ordinary room temperature. The role of adrenal hormones was also studied in view of the findings of Fawcett and Jones (1949) that the functional integrity of the adrenal cortex is essential for the normal complement of lipids in brown adipose tissue. It has also been reported that during stress, changes in the weight and appearance of this tissue parallel changes in the adrenals (Selye and Timiras, 1949; Lemonde and Timiras, 1951.)

MATERIAL AND METHODS

Young albino male rats of the Wistar strain were used. They were housed in individual cages and fed a purified ration supplemented with crystalline vitamins. This ration contained 18% protein in the form of casein and 4% fat (Mazola corn oil). Thyroid powder (VioBin), when used, was incorporated in the ration at the level of 0.05%. Hormones were administered subcutaneously in the following daily doses: thyroxine-sodium (BDH) 5 mg./kg. of body weight; cortisone acetate (Merck), 2.5 mg.; DCA (Ciba), 2.5 mg; and ACTH (Armour), 2 mg. Thyroxine-sodium was dissolved in dilute sodium hydroxide and 0.9% NaCl was used as a diluent for other hormones when indicated.

Bilateral adrenalectomy was performed in a single sitting through two cutaneous incisions under ether anesthesia. Adrenalectomized rats were given tap water containing 1% sodium chloride *ad libitum.* The animals were sacrificed by decapitation under light ether anesthesia and the interscapular brown adipose tissue was immediately dissected and weighed. It was dried to constant weight *in vacuo* at 48° C. The dry matter was later homogenized in a chloroform-methanol mixture and the fat was extracted therefrom at

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room temperature according to the method of Folch *et al.* (1951). The fresh weight of the perirenal fat (left side) was used as an index of fat depots, Pagé and Babineau (1951) having found the amount of fat in this tissue to be a reliable guide to total body fat.

Statistical treatment was limited to the determination of the standard error by the usual formula where it is equal to $\sqrt{\Sigma d^2/n\ (n-1)}$; $n(n-2)$ was used in groups numbering less than ten animals. The probability of identity between values was calculated from standard tables for Student's t.

RESULTS

Thyroid feeding

Thyroid feeding to young rats for a period of two weeks led to a considerable hypertrophy of the brown adipose tissue. This gain in the fresh weight of the tissue could be entirely accounted for by the increase in fat content since no change occurred in the water or fat-free dry matter by comparison with control rats killed on the same day (Table 1, Experiment 1). It is also seen that fat deposition in the brown adipose tissue was accompanied by a reduction of depot fat as reflected in the size of the perirenal fat. On the other hand, body growth in untreated animals results in a significant increase in all three fractions of the brown fat. This is the general picture which was invariably reproduced in all experiments of this type.

TABLE 1. EFFECT OF THYROID FEEDING ON THE BROWN ADIPOSE TISSUE OF INTACT AND OF ADRENALECTOMIZED RATS

Group	Experiment 1*					Experiment 2†	
	Intact rats			Adrenalectomized rats		Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed	Controls (end of expt.)	Thyroid fed
No. rats	10	10	10	11	10	6	7
Mean body wt., gms.							
Initial	145	145	145	145	147	236	226
Final	—	208	183	147	150	302	266
Perirenal fat, mg.	569 ± 50	942 ± 110	410 ± 60	negligible	negligible	1191 ± 300	667 ± 300
P‡		.01	.01				.7
Brown adipose tissue							
Fresh wt., mg.	167 ± 14	284 ± 20	407 ± 21	163 ± 12	197 ± 19	350 ± 32	554 ± 69
P		.01	.01	(.9)§	.25		.02
Water content, mg.	75 ± 5	130 ± 11	130 ± 7	108 ± 9	109 ± 6	172 ± 16	190 ± 9
P		.01	.9	(.01)	.9		.3
Fat content, mg.	60 ± 8	113 ± 15	234 ± 20	24 ± 5	56 ± 15	124 ± 17	313 ± 26
P		.01	.01	(.01)	.05		.01
Fat-free dry matter content, mg.	32 ± 3	41 ± 3	43 ± 5	31 ± 2	32 ± 4	54 ± 7	51 ± 13
P		.05	.8	(.8)	.8		.8

* Experimental period: 2 weeks.
† Experimental period: 36 days; rats thyroid fed during the last 29 days.
‡ P = Probability of identity between the two values shown above.
§ Probability of identity between untreated adrenalectomized rats and controls killed initially.

The effect of adrenalectomy and of thyroid feeding to adrenalectomized rats was studied concurrently. In the case of the non-treated animals, the size of the brown fat at the termination of the experimental period was the same as that of control rats killed initially. Its composition was significantly altered, however, the fat content dropping markedly and being replaced by an equal quantity of water (Table 1, Experiment 1). It might be argued that the stationary weight of the brown adipose tissue was consequent upon the lack of gain in body weight of these animals. However, a similar growth inhibition of this tissue was observed in a subsequent experiment where the adrenalectomized rats registered substantial gains in body weight (Table 2). The results seem to confirm fully the conclusion reached by Fawcett and Jones (1949) and mentioned above.

TABLE 2. EFFECT OF DCA ON THE BROWN ADIPOSE TISSUE OF INTACT AND ADRENALECTOMIZED RATS

Group	Intact rats			Adrenalectomized rats	
	Controls (beginning of expt.)	Controls (end of expt.)	DCA*	Controls (end of expt.)	DCA*
No. rats	10	10	10	12	12
Mean body wt., gms.					
Initial	140	141	140	140	140
Final	—	231	226	202	218
Perirenal fat, mg.	277 ±35	1078 ±156	1148 ±159	389 ±95	549 ±60
P		0.01	.7		.2
Brown adipose tissue:					
Fresh wt., mg.	248 ±20	291 ±21	291 ±16	245 ±18	244 ±16
P		.2	.9		.9
Water content, mg.	—	142	126	126	99
Fat content, mg.	—	101	120	74	100
Fat-free dry matter content, mg.	—	48	45	45	45

* Administered during the last two weeks of a three week experimental period.

Thyroid feeding to adrenalectomized rats caused a slight but not significant increase in the fresh weight of the brown fat (Table 1, Experiment 1). This increase was confined to the fat fraction, however, and on this basis it is significant at the 5% level. Identical results were later obtained in adrenalectomized rats receiving thyroxine subcutaneously (Table 3). In contrast to these findings, when larger rats were adrenalectomized and fed thyroid powder for an extended period of time, there resulted a hypertrophy of the brown fat (Table 1, Experiment 2) comparable in degree and nature to that observed in intact rats similarly treated. It is doubtful if one can exclude in such a prolonged treatment the possible intervention of accessory adrenal tissue or even the cortical-like action of androgens. These same rats were also reasonably well supplied with fat reserves in contrast to the previous ones so that in the smaller rats, limited response to thyroid feeding or to thyroxine administration may have been partly due to lack of mobilizable fat.

Desoxycorticosterone acetate

Results shown in Table 2 indicate that the administration of DCA has no effect on the size of the brown adipose tissue either in intact or in adrenalectomized rats, nor does it affect significantly the amount of depot (perirenal) fat. Analyses of the brown fat were carried out on pooled samples so that the significance of any observed difference cannot be evaluated statistically. It may be noted, however, that in both treated groups there occurs a rise in the fat content of the brown adipose tissue and a concomitant decrease in water, a unique finding in all our studies to date.

Cortisone and thyroxine in adrenalectomized rats

The effect of cortisone in adrenalectomized rats receiving thyroxine or not was next investigated. Adrenalectomized controls received no treatment or were given thyroxine alone (Table 3). It is seen that cortisone alone caused a significant increase in the size and fat content of the brown adipose tissue as compared to the untreated controls. In fact, this hypertrophy is as large as that observed following thyroxine or cortisone treat-

TABLE 3. EFFECT OF THYROXINE, CORTISONE, AND OF THYROXINE+CORTISONE ON THE BROWN ADIPOSE TISSUE OF ADRENALECTOMIZED RATS

Group	Control	Thyroxine	Cortisone	Thyroxine + cortisone
No. rats	10	6	10	10
Mean body wt., gms.				
Initial	115	119	115	116
Final	135	125	132	115
Perirenal fat, mg.	88 ± 20	negligible	215 ± 30	112 ± 20
P vs controls			.01	.4
Brown adipose tissue				
Total fresh weight, mg.	254 ± 18	309 ± 25	403 ± 19	546 ± 42
P vs controls		.1	.01	.01
P vs cortisone group		.01		.01
Water content, mg.	152 ± 9	162 ± 6	169 ± 7	174 ± 10
P vs controls		.1	.1	.3
Fat content, mg.	62 ± 8	99 ± 17	182 ± 16	316 ± 33
P vs controls		.05	.01	.01
P vs cortisone group		.01		.01
Fat-free dry matter content, mg.	40 ± 5	48 ± 4	52 ± 6	56 ± 5
P vs controls		.1	.07	.02

Experimental period: 14 days; thyroxine or cortisone given on the 9th, 10th and 11th day.

ment in intact rats (Tables 4 and 5). When given in conjunction with thyroxine, the increase in weight and fat content is double that obtained with cortisone alone.

These results would indicate that cortisone administration restores the action of thyroxine which is otherwise greatly impeded by adrenalectomy.

Cortisone and thyroxine in intact rats

In intact animals, the action of thyroxine is similar to that obtained by thyroid feeding (Tables 4 and 5). Cortisone has an effect comparable to that of thyroxine with this difference that the water content rises significantly and that the deposition of fat is less pronounced (Table 4). Seifter *et al.* (1951) have also reported an increase in the size and fat content of this tissue in pregnant rats given cortisone. On the other hand, Antopol (1950) working on mice and using relatively larger doses of cortisone observed an atrophy of the brown fat. Sala *et al.* (1951) report that this tissue regresses under the influence of cortisone administered over an extended period of time.

TABLE 4. EFFECT OF THYROXINE AND OF CORTISONE ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Cortisone
No. rats	20	10	8
Mean body wt., gms.			
Initial	135	137	135
Final	170	149	140
Perirenal fat, mg.	436 ±37	288 ±37	328 ±50
P vs controls		.02	.1
Adrenal wt., mg.	23.7 ±0.6	26.6 ±0.6	19.0 ±1.5
P vs controls		.01	.01
Brown adipose tissue:			
Total fresh wt., mg.	326 ±19	461 ±26	426 ±27
P vs controls		.01	.01
P vs thyroxine group			.4
Water content, mg.	156 ±7	157 ±9	177 ±7
P vs controls		.9	.05
Fat content, mg.	124 ±12	254 ±17	190 ±17
P vs controls		.01	.01
P vs thyroxine group			.02
Fat-free dry matter content, mg.	46 ±3	50 ±4	59 ±6
P vs controls		.4	.08

Experimental period: 6 days; thyroxine or cortisone given on the 1st, 2nd and 3rd day.

When cortisone is given in conjunction with thyroxine, an apparently

TABLE 5. EFFECT OF THYROXINE, THYROXINE+CORTISONE AND OF ACTH ON THE BROWN ADIPOSE TISSUE OF INTACT RATS

Group	Control	Thyroxine	Thyroxine + cortisone	ACTH
No. rats	12	12	12	12
Mean body wt., gms.				
Initial	134	134	134	134
Final	174	153	132	171
Perirenal fat, mg.	411 ±50	90 ±10	100 ±10	421 ±60
P vs controls		.01	.01	.9
Adrenal wt., mg.	23.7 ±0.7	28.1 ±1.2	18.6 ±1.4	25.8 ±0.7
P vs controls		.01	.01	.08
Brown adipose tissue				
Total fresh wt., mg.	310 ±15	441 ±15	544 ±29	367 ±22
P vs controls		.01	.01	.05
P vs thyroxine group			.01	
Water content, mg.	156 ±4	151 ±5	161 ±9	174 ±9
P vs controls		0.5	0.6	0.1
P vs thyroxine group			.3	
Fat content, mg.	107 ±11	240 ±9	333 ±19	134 ±14
P vs controls		.01	.01	.2
P vs thyroxine group			.01	
Fat-free dry matter content, mg.	47 ±1	50 ±3	50 ±6	59 ±4
P vs controls		.3	.6	.01

Experimental period: 8 days; Rats injected daily, the 6th day excepted.

additive response is obtained (Table 5). It is to be noted that cortisone induces an atrophy of the adrenals even in the presence of excess thyroxine. The fact that this hormone is equally potent whether the adrenals are removed or atrophied points to its preponderant role among cortical hormones with respect to fat deposition in the brown adipose tissue.

ACTH in intact rats

The action of ACTH is reported in Table 5. The dosage used was too low to increase significantly the size of the adrenals. Nevertheless, the brown adipose tissue was significantly hypertrophied and a general increase in all three fractions occurred, the rise in fat-free dry matter content being statistically significant. This response differs somewhat from that obtained with thyroxine or cortisone and is more reminiscent of spontaneous changes occurring during body growth (Table 1). Baker, Ingle and Li (1950) have observed a higher fat content of the brown adipose tissue in rats treated for 21 days with ACTH. From the trend shown in Table 5, it can be assumed that a longer treatment would have yielded similar results.

Hormonal treatment and depot fat

With respect to general fat stores, it can be seen that hyperthyroidism led to a considerable decrease in the amount of perirenal fat in all cases. Cortisone, on the other hand, seemed to prevent the loss of fat consecutive to adrenalectomy although it did not affect body weight. It also afforded some measure of protection against fat depletion in thyroid-treated adrenalectomized rats. These results are in accordance with the findings of Stoerk and Porter (1950). In intacts rats, neither cortisone nor ACTH had a perceptible effect on depot fat and the same seems to apply to DCA in intact and in adrenalectomized animals.

DISCUSSION AND CONCLUSIONS

Of chief interest are the findings: (1) that thyroxine elicits a minimum response in the absence of the adrenals; (2) that cortisone is equally effective in intact and in adrenalectomized rats; and (3) that an enhanced response is obtained when the two hormones are combined either in intact or adrenalectomized animals. There thus appears to exist a synergism between cortisone and thyroxine in respect to fat deposition in the brown adipose tissue. It may be that excess thyroxine given alone exhausts the cortisone producing capacity of the adrenal while cortisone given singly inhibits the production of thyroid hormone as some reports indicate (Money *et al.*, 1951; Lederer, 1952).

The origin of this extra fat in the brown adipose tissue remains to be determined. Experiments now underway seem to indicate that it is mobilized from fat stores in other regions. In view of the reported antilipogenic action of cortisone, one would hardly expect this fat to have been synthesized *in situ.* It is difficult to correlate our findings with those of Engel and Scott (1951) regarding the effect of cortisone on glycogen deposition in the brown fat. These authors express the glycogen concentration on a fat basis and should cortisone have stimulated fat deposition, it would follow that glycogen concentration would suffer an apparent decrease unless the whole tissue content in this substance rose proportionately.

Glycogen deposition in the brown adipose tissue is often considered as indicative of fat synthesis (Wertheimer and Shapiro, 1948). However, if the fat laid down in the brown adipose tissue originates elsewhere, one may ponder whether it is later oxidized directly or over the pathway of carbohydrate synthesis. Ingle (1950) has envisaged the possibility of fat conversion to carbohydrate under the influence of corticosteroids and Segaloff and Many (1951) suggest that extra urinary glucose in phlorizinized rats may have arisen from fat. ACTH and cortisone were among the compounds having a marked effect on gluconeogenesis and cortisone was much less ketogenic than other such substances. Wells and Kendall (1940), in similar studies, had already reported a synergism between the actions of Compound E and of the thyroid gland so that the full exhibition of either was dependent upon the presence of adequate amounts of the other hormone. The similar interrelationship between cortisone and thyroxine in respect of gluconeogenesis and of fat deposition in the brown adipose tissue is striking although no link between the two processes has been shown to exist.

SUMMARY

1. Thyroxine and cortisone both promote the accumulation of fat in the brown adipose tissue of intact rats and their actions appear to be synergistic. Little or no change occurred in the water and fat-free dry matter contents.

2. In adrenalectomized animals, the response to thyroid hormone was minimal while the action of cortisone administered alone or in conjunction with thyroxine was comparable to that obtained in intact rats.

3. The administration of DCA caused no change in the size of the brown fat.

4. ACTH caused a slight hypertrophy of the brown adipose tissue, characterized by increases in all three fractions studied.

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A SODIUM-EXCRETING EFFECT OF DESOXYCORTICOSTERONE IN ADRENALECTOMIZED MICE*

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METHODS recently reported for the bioassay of desoxycortisosterone (DOC) (Dorfman, 1947; Dorfman, 1949; Deming and Luetscher, 1950; Spencer, 1950; Simpson and Tait, 1950, 1952: Luetscher and Demng, 1951; Marcus, Romanoff and Pincus, 1952) have utilized the action of DOC in adrenalectomized animals in decreasing urinary excretion of sodium, in increasing the urinary excretion of potassium or in effecting a combination of these actions. Little quantitative information has been published regarding the optimal sodium loading of the animals used for bioassay. Workers employing isotopic techniques have favored a low sodium load (Dorfman, 1947; Simpson and Tait, 1952) while those using the alteration in the urinary excretion of sodium as determined chemically or by flame photometer have favored a high salt load (Luetscher and Deming, 1951; Spencer, 1950; Marcus, Romanoff and Pincus, 1952). Although much is known concerning the effect of water loading in adrenalectomized animals (Gaunt, 1944, 1950; Hays and Mathieson, 1945; Birnie, Eversole and Gaunt, 1948; Frost and Talmage, 1951) few of these results can be applied to determining the optimal water load for the bioassay of salt retaining steroids, because urinary sodium excretion was not adequately measured.

The present studies were initiated to define more precisely the optimal sodium and water loads in adrenalectomized mice used for the bioassay of DOC. During the course of the sodium loading experiments, DOC was found to have a sodium excreting effect in small doses, and the usual sodium retaining effect in larger doses. Strain differences in sensitivity to DOC were noted in respect to both sodium excretion and sodium retention.

METHODS

Method 1

Male mice, strain CF1 Carworth Farms and strain C57 BBF1 Roscoe B. Jackson

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Memorial Laboratory, weighing from 20 to 25 grams were adrenalectomized using a single dorsal skin incision. These mice were maintained postoperatively on 0.9% sodium chloride solution, hereafter called saline, and "Friskies" dog biscuit containing 2.8 mg. sodium and 4.8 mg. potassium per gram of biscuit. Tests were performed from the fourth to the fourteenth post-operative day. Sixteen hours before assay solid food was removed and the animals were given a 10% solution of glucose or sucrose to drink. Desoxycorticosterone (free alcohol) dissolved in 10% ethanol was used throughout these studies. The control mice received 0.2 ml. 10% ethanol intraperitoneally, approximately 20 minutes before the assay was begun. The appropriate dose of sodium loading solution was calculated on the basis of body weight and injected subcutaneously using a number 25, two inch needle. In addition to sodium chloride the loading solution contained 5% glucose and four m.eq. per liter potassium. Urine was forcibly expressed by manual pressure over the bladder and the animals were placed in individual 600 ml. beakers with $\frac{1}{4}$ inch wire screen platforms over the bottom of the beakers. At the end of 5 hours the animals were removed and any feces present were discarded. Urine was again forcibly expressed from the bladder into the beakers. The contents of the beakers were transferred to volumetric flasks using four washes of distilled water. The total amounts of sodium and potassium in the urine were then determined with the Weichselbaum-Varney flame photometer.

Method 2

It was noted that the physical condition of the mice was often poor using Method 1 and the mortality during assay occasionally was 10–20%. For this reason the following modification was investigated. Nineteen hours before assay solid food was omitted and a solution of 10% glucose in 0.9% saline was used for drinking. Three hours before assay the mice were given only 10% glucose to drink. During the 5 hour assay period a cube of table sugar was placed in each beaker. Strain BBF1 mice ate the sugar very well, but strain CF1 mice largely ignored it. The sugar used contained approximately 0.06 mg. of sodium and 0.01 mg. of potassium per gram. In view of the small amount of sugar contaminating the urine, this source of error can be overlooked.

RESULTS

Strain CF1 Mice, Method 1

Mice of this strain were given loading solutions containing 0.5 mg., 0.3 mg., 0.1 mg. and 0.025 mg. of sodium chloride per gram of body weight administered subcutaneously in a volume of 0.5 ml. To observe the effect of the volume of loading solution on sodium excretion and DOC sensitivity the 0.5 mg. per gram, 0.1 mg. per gm. and 0.025 mg. per gram doses of sodium chloride were also injected in a volume of 2 ml. The excretion of sodium per gram of body weight under these experimental conditions with and without DOC are presented graphically in Figure 1. The decrease of urinary sodium produced by DOC was most evident with 0.5 mg. per gram of weight in a volume of one half ml. With this load a significant decrease in sodium excretion was apparent with 10 μg. of DOC ($P=0.007$). When the same amount of sodium chloride was given in a volume of 2 ml. a dose of 25 μg. of DOC was required to establish a significant decrease in sodium

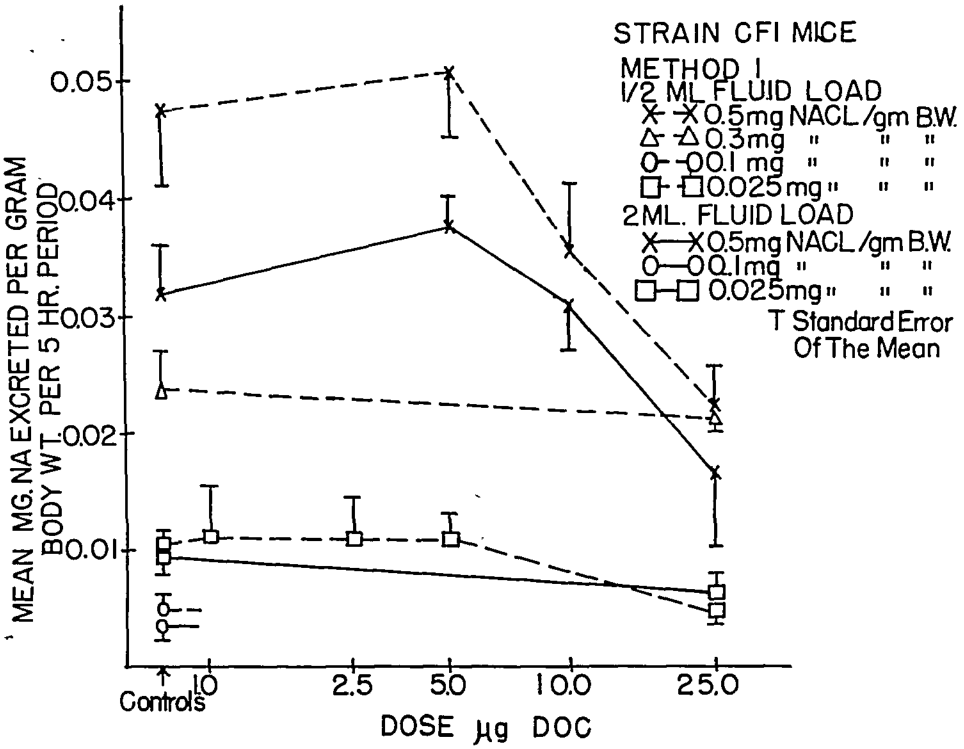

FIG. 1. The urinary sodium excretion in the strain CF1 mouse and its response to desoxycorticosterone with various fluid and NaCl loads. Standard error of the mean indicated in only one direction to avoid overlap. Each point represents the mean of from 8 to 35 animals.

excretion. With sodium loads less than 0.5 mg. of NaCl per gram of weight the control sodium excretion was correspondingly reduced. The absolute decrease in sodium excretion following DOC under these circumstances was small and too variable to be useful for assay. From these results pretreatment with 0.5 mg. NaCl per gram of body weight in 0.5 ml. was the most favorable condition for demonstrating the salt-retaining action of DOC.

Strain CF1 Mice, Method 2

Allowing adrenalectomized mice access to saline drinking water until 3 hours before assay in effect increases the salt load. The excretion of sodium with and without DOC under these conditions is presented graphically in Figure 2. Urinary sodium actually increases with doses of 5 and 10 µg. of DOC. Not until a dose of 25 µg. of DOC was given did a fall in urinary sodium from the maximum value occur. A statistical analysis of these results is presented in Table 1.

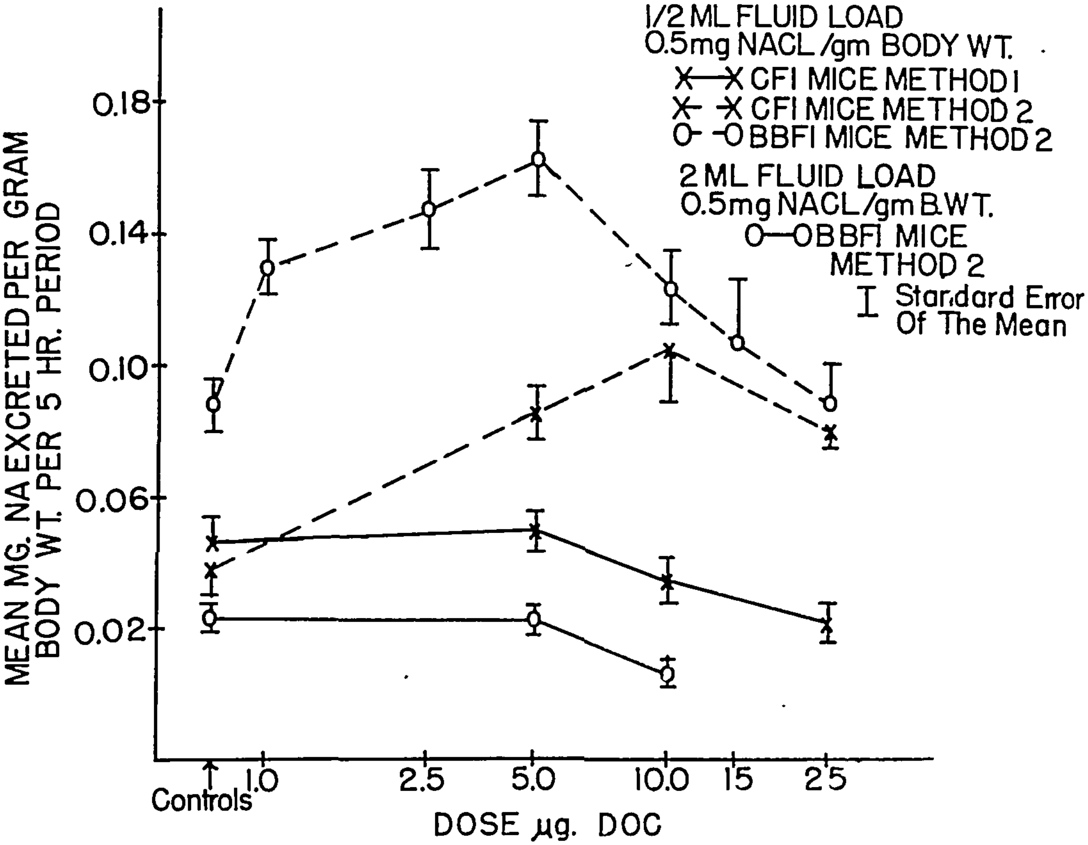

FIG. 2. The comparative effect of desoxycorticosterone on the urinary sodium excretion of strain CF1 mice and strain BBF1 mice. Standard error of the mean indicated in only one direction at times to avoid overlap. Note different scale from figure 1.

Strain BBF1 Mice

To find mice more sensitive to DOC than the CF1 strain, mice of the C57 BBF1 strain were tested. These mice tolerated sodium restriction very badly and when tested with Method 1, the mice were in such poor condition that sodium excretion was always very low regardless of fluid or sodium load used. For this reason studies on this strain are limitd to Method 2. With a sodium load of 0.5 mg. NaCl per gm. body weight and a fluid load

TABLE 1. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN CF1 MICE

	Controls	5 µg. DOC	10 µg. DOC	25 µg. DOC
No. of Animals	8	13	11	8
Mean mg. Na Excr. per gm. body wt. per 5 hrs.	0.0386	0.0857	0.1053	0.0806
Stan. error of mean	±0.0087	±0.0086	±0.0150	±0.0059

of 0.5 ml. DOC had a pronounced sodium excreting effect (Fig. 2). Maximal sodium excretion occurred with 5 µg. DOC in contrast to the strain CF1 mice which required 10 µg. DOC for maximal sodium excretion. A nearly linear fall in sodium excretion occurred when plotted against the logarithm of the dose at levels of 10 µg., 15 µg. and 25 µg. doses of DOC in the BBF1 mice. (The numerical values are analyzed in Table 2.) When the same amount of sodium chloride was given in a volume of 2 ml. (Fig. 2) no increased excretion of sodium occurred with small doses of DOC. The promotion of sodium excretion by 5 µg. of DOC decreased markedly with increasing fluid load (Fig. 3).

TABLE 2. THE SODIUM EXCRETING AND RETAINING EFFECT OF DESOXYCORTICOSTERONE IN STRAIN BBF1 MICE

	Control	1 µg. DOC	2.5 µg.	5.0 µg.	15 µg.	15 µg.	25 µg.
No. of Animals	22	10	8	20	20	8	11
Mean mg. Na excr. per gm. body wt. per 5 hr. period	0.0884	0.1309	0.1480	0.1635	0.1256	0.1083	0.0897
Standard error of mean	± .0088	± .0083	± .0137	± .0102	± .0112	± .0192	± .0138

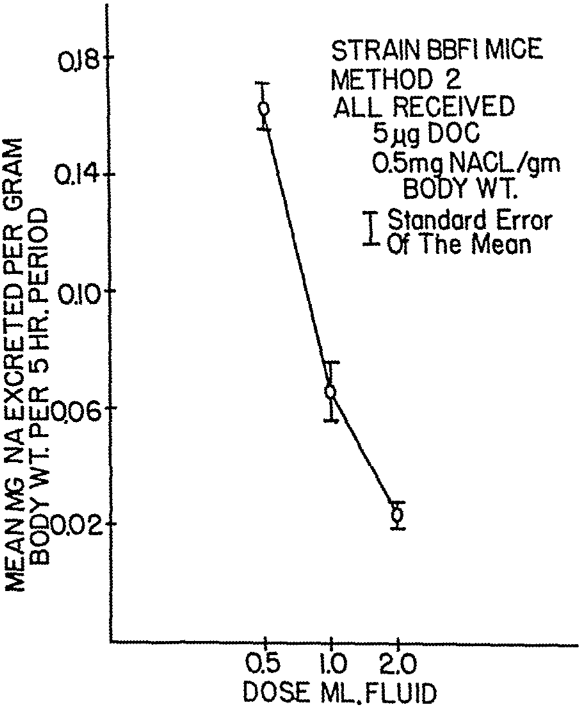

FIG. 3. The effect of increasing fluid load on the maximal urinary sodium excretion of BBF1 mice produced by 5 µg. DOC. Each point represents the mean of from 7 to 20 animals.

To minimize intestinal absorption of exogenous sodium, the drinking water containing sodium chloride was removed 5 hours rather than 3 hours before study. The control sodium excretion was somewhat lower in these experiments with a mean of 0.0601 mg. per gm. body weight per 5 hours, but increased excretion of sodium again occurred with 5 μg. DOC, the mean being 0.1006 mg. per gm. body weight per 5 hours.

Not only did the total amount of sodium but also the concentration of urinary sodium increase in strain BBF1 mice given 5 μg. DOC. Thus at the beginning of the assay 3 hours after removal of saline drinking water, the mean sodium concentration of the urine was 243 m.eq. per kilogram of urine (S.E. = ±41). Three hours after receiving 5 μg. DOC the mean sodium concentration of the urine was 424 m.eq. per kilogram of urine (S.E. = ±45). This difference is significant ($P = 0.01$). Urinary excretion of potassium was not significantly altered by doses of DOC up to 25 μg.

DISCUSSION

Speirs *et al.* (1951) were able to demonstrate that the strain of mice C57 BBF1 was about ten times more sensitive to cortisone as judged by eosinophil response than other strains tested. Our results indicate a comparable sensitivity to DOC in this same strain. These mice require 5 μg. DOC to reach maximal sodium excretion while strain CF1 mice required 10 μg. DOC for maximal sodium excretion. Significant sodium retention occurred in strain BBF1 mice with 10 μg. DOC, but strain CF1 mice required 25 μg. DOC for significant sodium retention. It would appear that mice of the C57 BBF1 strain are two to three times more sensitive to DOC than the mice of the CF1 strain.

The use of the C57 BBF1 strain for the bioassay of DOC seems possible under the conditions specified because of the wide differences in urinary sodium with doses of DOC from 1 μg. to 25 μg. The biphasic character of sodium excretion with DOC would necessitate testing several dosage levels of the material to be assayed in order to locate the value on the ascending or descending slope of the curve of urinary sodium excretion.

The promotion of sodium excretion in our experiments was unexpected. However, desoxycorticosterone has been shown to cause sodium excretion in Cushing's syndrome (Soffer, Gabrilove and Jacobs, 1949) and in normal dogs receiving high salt loads (Green, Farah and Klemperer, 1950). Large doses of desoxycorticosterone glycoside intravenously in humans with presumably intact adrenals causes sodium excretion, but with a fall in urine sodium concentration (Green *et al.*, 1950). The nearly twofold increase in control sodium excretion effected by 5 μg. DOC in adrenalectomized mice of the BBF1 strain could be largely attributed to increased urinary sodium concentration. Gastrointestinal absorption of sodium appears to contribute little to the sodium diuresis produced by DOC. Sodium excretion with 5 μg. DOC increased approximately to the same degree after animals had been off saline drinking-water for 5 hrs. Increasing the subcutaneous fluid load decreased or abolished the sodium diuresis produced by DOC.

It is known that dogs (Roemmelt, Sartorius and Pitts, 1949) and rats (Friedman, 1948; Lotspeich, 1949) maintain a normal glomerular filtration rate for a period of time after adrenalectomy when adequately hydrated. Animals used in this study had free access to saline solution and were used for a maximum period of seventeen days after adrenalectomy. It is suggested, therefore, that increasing glomerular filtration rate was probably not the mechanism whereby DOC caused increased sodium excretion in the present experiment. Adrenalectomized dogs and patients with Addison's disease have a reduced capacity to excrete sodium under a high sodium load due to increased tubular reabsorption of sodium (Roemmelt, Sartorius and Pitts, 1949; Burnett, 1951). The sodium diuresis in the present experiments would seem to be due to a depression of tubular reabsorption of sodium with small doses of DOC, thus restoring to the adrenalectomized animal a more normal ability to excrete high sodium loads. As the dose of DOC is increased the depression of tubular reabsorption of sodium with small doses of DOC is followed by the more familiar increase in tubular reabsorption of sodium. The data at hand do not permit elucidation of the mechanism of depression of tubular reabsorption of sodium by DOC.

SUMMARY

The optimal conditions for demonstrating the action of DOC in adrenalectomized mice have been studied. A sodium load of 0.5 mg. NaCl per gm. of body weight proved most satisfactory. DOC in small doses promoted sodium excretion under high sodium loads while sodium retention occurred with larger doses of DOC. Adrenalectomized mice of the BBF1 strain are two to three times more sensitive to DOC than mice of CF1 strain.

The increased sodium excretion with small doses of DOC could be accounted for by increased urine concentration of sodium. The sodium diuresis can best be explained by decreased tubular reabsorption.

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EFFECTS OF ANDROGENS ON DEHYDROGENASE SYSTEMS

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WE RECENTLY reported an inhibitory effect on the succinoxidase system of rat liver homogenates by certain water-soluble androgens (Kalman, 1952). There was some evidence in this work that succinic dehydrogenase was the target of the inhibitor. Because different dehydrogenases respond similarly to a number of inhibitors it was decided to try the effect of the androgens on the malic dehydrogenase system. In addition certain observations regarding kinetics of succinoxidase inhibition are included in this report.

METHODS

Adult white rats of the Slonaker-Wistar strain were used. No sex differences in regard to the work reported were noted and males and females were used interchangeably. The animals were killed by decapitation, and appropriate amounts of liver tissue were removed, weighed, and homogenized in cold, distilled water. Homogenates were prepared as described by Potter and Elvehjem (1936). For each enzyme system control runs were made to ensure that optimal amounts of all components were present. Control QO_2 values fell within the range of conventional results. Calculations of oxygen consumption were based on time periods during which control vessels showed essentially linear oxygen uptake. The gas phase for all systems was oxygen. In addition to the 3.0 ml. of reaction mixture, 0.1 ml. of 5% KOH was placed on filter paper in the center well of each vessel.

For the malic dehydrogenase assay the method of Potter (1946) was used. The Warburg vessels contained 0.3 ml. each of 0.5 M malate, 0.5 M glutamate, 1% diphosphopyridine nucleotide (DPN) solution, 0.0004 M cytochrome C, 0.8 ml. of 0.1 M PO_4 buffer at pH 7.4, inhibitor and homogenate in amounts specified below, and distilled water to make 3.0 ml. The DPN was added from the sidearm at the start of the reaction. Nicotinamide was omitted because it did not enhance oxygen consumption over the time periods employed.

The succinoxidase system used was that described in Umbreit's text (1949). The reaction vessel contained 0.3 ml. each of 0.004 M $CaCl_2$, 0.004 M $AlCl_3$, 0.0001 M cytochrome C, 0.5 M succinate, 1.0 ml. of 0.1 M PO_4 buffer at pH 7.4, and inhibitor as described below. A 5% liver homogenate was prepared in the usual way and then centrifuged for 10 minutes at 600×gravity. Homogenate from the supernatant was added to reaction vessels which had been filled with the other reactants and chilled for several minutes.

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The androgens used were sodium androsterone sulfate, sodium testosterone sulfate, and testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). These steroids were used in aqueous solutions. The sodium testosterone sulfate and sodium androsterone sulfate are water soluble at pH 7.4. Testosterone 17-β diethylaminoethylcarbonate hydrochloride is insoluble at pH 7.4 (the pH of the reaction vessels) and above, forming a very fine suspension in an aqueous medium. This suspension may be dissolved by bringing the pH back to neutrality or to the acid side. During the course of this work some diethylaminoethanol hydrochloride was made available through the courtesy of Dr. E. Oppenheimer. This compound was tested for possible *in vitro* effect since it forms the major part of the side chain in "soluble" testosterone.

RESULTS

Inhibition of the malic dehydrogenase system by small concentrations of "soluble" testosterone is shown in Table 1. Sodium testosterone sulfate proved to be much less effective an inhibitor as indicated by the concentrations required. Sodium androsterone sulfate was not available for test on the malic dehydrogenase system.

TABLE 1. EFFECT OF WATER SOLUBLE ANDROGENS ON THE MALIC DEHYDROGENASE SYSTEM OF RAT LIVER

(QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.)

Homogenate mg. dry weight per vessel	Molar concentration		QO_2	Per cent inhibition
	Sodium testosterone sulfate	"Soluble" testosterone		
1.5	—		98	Control
1.5	3.4×10^{-4}		82	16.3
1.5	6.8×10^{-4}		46.8	52.3
1.5	1.0×10^{-3}		26.0	75.0
1.5	1.4×10^{-3}		14.0	86.0
1.5	1.7×10^{-3}		16.0	84.0
3.0		—	105	Control
3.0		1.8×10^{-5}	100	4.8
3.0		2.7×10^{-5}	90	14.2
3.0		3.6×10^{-5}	46	56.0
3.0		4.5×10^{-5}	23	78.0
3.0		5.4×10^{-5}	21	80.0
3.0		6.3×10^{-5}	17	84.0
3.0		7.2×10^{-5}	12	88.5

In Table 2 data are presented which show inhibition of the succinoxidase system by "soluble" testosterone, by sodium androsterone sulfate, and by sodium testosterone sulfate. The inhibitory effect of these compounds as shown by the concentrations used was most pronounced with "soluble" testosterone, less with sodium androsterone sulfate, and least with sodium testosterone sulfate. Diethylaminoethanol hydrochloride does not inhibit succinoxidase in concentrations of 3.5×10^{-5} molar. Such amounts are equivalent to the amount of this compound which is present in the side chain of "soluble" testosterone at concentrations of 9×10^{-5} molar.

TABLE 2. EFFECT OF WATER SOLUBLE ANDROGENS ON THE SUCCINOXIDASE SYSTEM OF RAT LIVER

QO_2 = c.mm. of oxygen uptake per mg. dry tissue per hour at 37.5° C.
All vessels contained 3.0 mg. dry weight of homogenate.
Concentrations of drugs are expressed in final molar concentration

Sodium testosterone sulfate	QO_2	Per cent inhibition	Sodium androsterone sulfate	QO_2	Per cent inhibition
—	76.0	Control	—	86	Control
3.4×10^{-4}	45.0	41.0	9×10^{-5}	81.5	18.9
6.8×10^{-4}	32.0	58.0	1.3×10^{-4}	68.0	26.7
1.0×10^{-3}	13.8	81.5	1.8×10^{-4}	61.0	27.8
1.4×10^{-3}	4.0	95.0	2.3×10^{-4}	60	33.4
1.7×10^{-3}	5.0	94.0	2.7×10^{-4}	57.4	62.3
2.0×10^{-3}	2.6	96.0	3.2×10^{-4}	34.7	92
			3.6×10^{-4}	0	100

"Soluble" testosterone	QO_2	Per cent inhibition	diethylamino-ethanol · HCl	QO_2	Per cent inhibition
—	74	Control	—	87	Control
$9\ \times10^{-6}$	66	10.8	3.5×10^{-5}	91	—
1.8×10^{-5}	36	51.5	3.5×10^{-5}	94	—
2.7×10^{-5}	14	81.0			
3.6×10^{-5}	8	89.0			
4.5×10^{-5}	3	96.0			
5.4×10^{-5}	2	97.5			

Preliminary results suggest that the water-soluble androgens also have an inhibitory effect on the lactic dehydrogenase system.

The curves of Figure 1 were derived from the data in Table 1 by plotting per cent inhibition of oxygen consumption by "soluble" testosterone for both malic and succinic dehydrogenase systems. Similar curves are obtained for inhibition by the sodium salt of testosterone sulfate of the malic dehydrogenase system and for the inhibition by sodium androsterone sulfate of the succinoxidase system. The inhibition of succinoxidase by sodium testosterone sulfate yields an atypical curve, no explanation for which is at hand.

Incubation time had no apparent effect on the inhibition of succinoxidase in liver homogenate by "soluble" testosterone in an experiment in which succinate was added from the side arms into the main chamber of the Warburg vessels at successive time intervals.

Figure 2 shows the effect of added homogenate on the testosterone inhibition of succinoxidase. Data are plotted according to Ackermann and Potter (1949). At each inhibitor concentration the same amount of enzyme is "bound" regardless of the amount of homogenate present.

DISCUSSION

The most interesting observation noted was the inhibition of the malic dehydrogenase system by "soluble" testosterone and by sodium testosterone sulfate. Erway *et al.* (1947) have reported that sodium androsterone

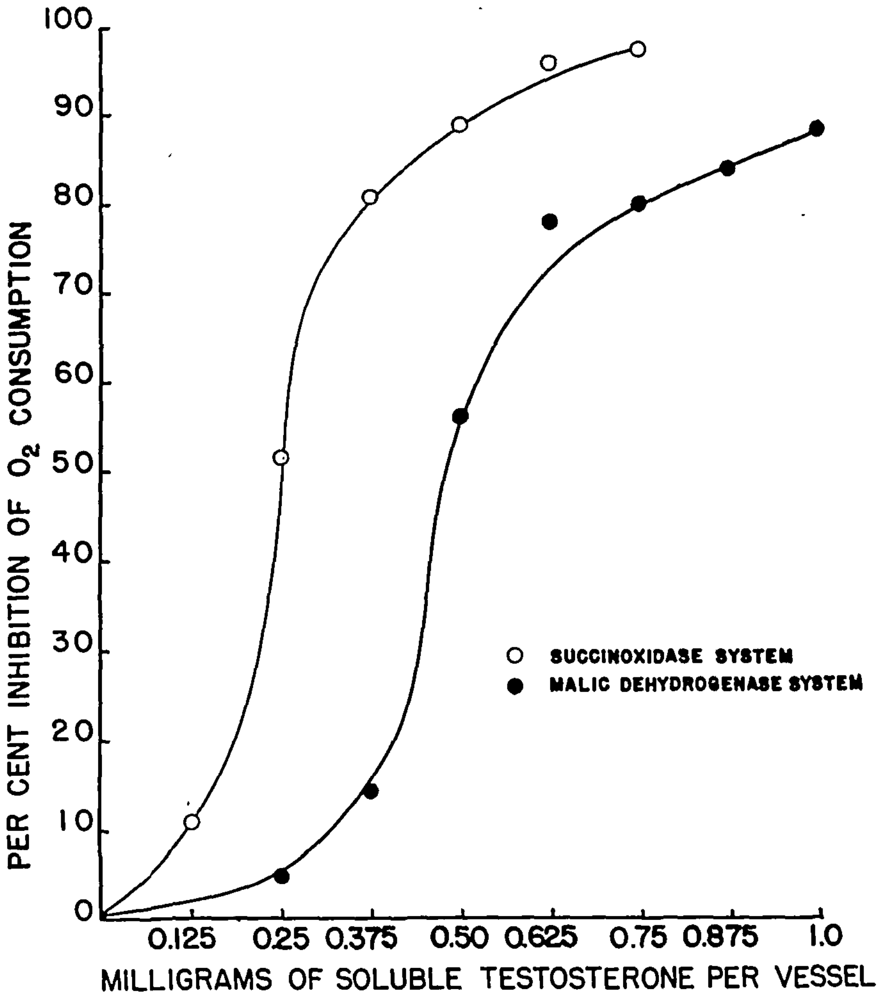

FIG. 1. The effect of concentration of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone) on the malic and succinic dehydrogenase systems. 0.125 mg. "soluble" testosterone is equivalent to 9×10^{-6} molar concentration.

sulfate has no significant effect on this enzyme system. They used inhibitor concentrations of 10^{-4} molar.

The inhibition of the succinoxidase and malic dehydrogenase systems by water-soluble steroids used appeared not to fit classical formulations (Lineweaver and Burk 1932). However, the data are compatible with the "irreversible" type of inhibitor described by Ackermann and Potter (1949). A definite reduction in oxygen consumption is effected by a given increment of inhibitor and is not reversed by increasing the amount of enzyme present (Fig. 2). Bain (1949) has demonstrated one instance of this type of inhibition in the effect of certain phosphate esters (diisopropylfluorophosphate, tetraethylpyrophosphate) on cholinesterase. Hayano and Dorfman (1951) showed a similar relationship for the inhibition of a d-amino acid oxidase by desoxycorticosterone.

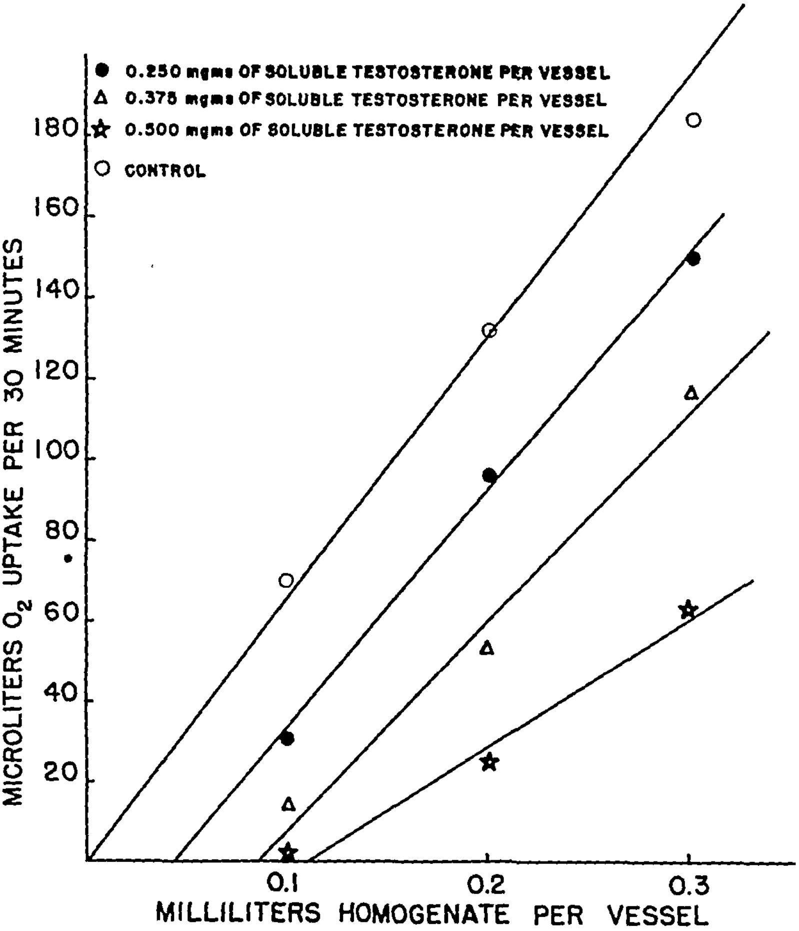

FIG. 2. The effect of increasing amounts of homogenate on the inhibition of the succinoxidase system by different amounts of testosterone 17-β diethylaminoethylcarbonate hydrochloride ("soluble" testosterone). 0.250 mg. soluble testosterone is equivalent to 1.8×10^{-5} molar concentration.

Dr. D. M. Greenberg suggested that the sigmoid shape of the curves relating per cent inhibition to inhibitor concentration (Fig. 1) resembles an adsorption isotherm. Such curves have been obtained, for example, for the amount of water vapor adsorbed on charcoal under conditions of varying pressure (Coolidge 1937). If we consider per cent inhibition analogous to inhibitor uptake, and inhibitor concentration analogous to "pressure," we may postulate that the steroid is adsorbed by the enzyme. The inhibition of saccharase by certain basic dyes has been described by Quastel and Yates (1936). When per cent inhibition was plotted against pH sigmoid curves similar to the ones plotted here were obtained. They suggested that the anion form of saccharase was better able to combine with basic dyes.

The significance of the variation in water solubility of the androgens mentioned above is not apparent. It may be that the long side chain of "soluble" testosterone makes this compound more susceptible to adsorption phenomena.

The site of action of the androgens on the dehydrogenase systems is thought to be the dehydrogenases themselves. Evidence for this has accumulated mainly by inference after other hydrogen carriers were bypassed or proved to be less susceptible to the inhibitors (Eisenberg *et al.*, 1949). A promising method of approach would seem to be fractionation of the components of the Warburg reaction mixtures in an attempt to recover the enzyme-inhibitor complex. Such an approach might also be used to study the enzyme-inhibitor "binding."

SUMMARY

The effects of water-soluble androgens on malic dehydrogenase and succinoxidase of rat liver homogenates were studied using conventional Warburg techniques. These substances were found to inhibit both enzyme systems in a manner suggesting an irreversible combination of enzyme and inhibitor. The data were also suggestive of some type of adsorption phenomenon involving enzyme and steroid.

Acknowledgements

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PATHWAYS OF ELIMINATION OF C^{14}-LABELED THYROXINE IN THE RAT[1]

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INTRODUCTION

TAUROG, Briggs, and Chaikoff (1951) using I^{131}-L-thyroxine demonstrated the presence of an unknown metabolic product, in addition to thyroxine, in the bile of rats. These investigators (1952) then reported that the thyroxine-containing derivative may be a glucuronide, but not a peptide. They further concluded that the conjugate is the primary metabolic product of endogenous thyroxine, and that the bile is the chief pathway of elimination for this hormone.

The present study is concerned with the elimination of DL-thyroxine-1-C^{14} in the bile, urine, and expired air. Further analysis of the bile has indicated that the thyroxine conjugate is not a peptide.

METHODS

DL-thyroxine-1-C^{14} used was that synthesized by Wang, Hummel, and Winnick (1952).[4] Its specific activity was 5.3 μc per mg. In all radioactivity measurements, the observed counts per minute were converted into equivalent μgm. of thyroxine, by reference to the known specific activity of the administered C^{14}-thyroxine. Samples were generally counted to within a probable error of 2 or 3%, and the values corrected for self-absorption. By way of illustration, 0.1 ml. of bile had of the order of 300 counts per minute, or 20 times background, with our counter.

Adult male Sprague-Dawley rats, fed ad libitum, were employed. The bile duct was cannulated with polyethylene tubing (inside diameter, 0.011 inch). When a good rate of flow was observed, the abdomen was closed, and the animal suspended in a restraining harness. Thyroxine was injected subcutaneously at a dose level of 780 μgm. (one μM) per kilo body weight. Bile samples were collected in 2 ml. portions over a 12 to 14-hour

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[4] The C^{14} for the synthesis of the thyroxine was obtained on allocation from the U. S. Atomic Energy Commission.

period. The radioactivity of the whole bile was determined by drying 0.1 ml. aliquots on aluminum plates, and counted in a thin mica window Geiger counter.

Paper chromatograms were performed as described by Taurog and co-workers (1951). Aliquots of 0.1 ml. bile were chromatographed immediately after the bile was collected. After the chromatogram was completed, the paper was cut into 1 cm. strips, and each strip extracted three times with 0.5 ml. of 0.01N NaOH. The extracts were air dried on aluminum plates for counting. Approximately 53% of the added thyroxine activity was recovered from the paper chromatograms.

The whole bile was subjected to the Van Slyke ninhydrin-carbon dioxide method (1941) which indicates the presence of free carboxyl and amino groups in amino acids. These determinations were performed at pH 2.5 on 0.1 ml. bile samples. The $BaCO_3$ was collected and counted as described by Winnick (1950).

A Benedict closed circuit metabolic apparatus was employed for the collection of the expired $C^{14}O_2$. The latter was removed from the expired air by bubbling the circulating gases through a 1N NaOH solution which was replaced at 2 or 3-hour intervals. Aliquots of the NaOH solutions were treated with excess $BaCl_2$, the precipitated $BaCO_3$ washed, dried, and plated for counting.

The concentration of C^{14} activity in the urine was determined on 0.1 ml. aliquots of samples, obtained at the various time intervals. The urine, which was collected from rats placed in metabolic cages, was plated as described for the bile.

RESULTS AND DISCUSSION

Figure 1 shows that the C^{14} of carboxyl-labeled DL-thyroxine is most rapidly eliminated by way of the bile. About 20% of the radioactivity was found in the bile in 12 hours. Almost identical results were obtained in 5 repeated experiments. Taurog and co-workers (1951) give a comparable value of 28% eliminated in 12 hours, for a subcutaneous injection of 330 μgm. of I^{131}-labeled L-thyroxine per kilo.

The appearance of 10% of the C^{14} in respiratory carbon dioxide in 12 hours indicates that the alanine portion of the thyroxine molecule is catab-

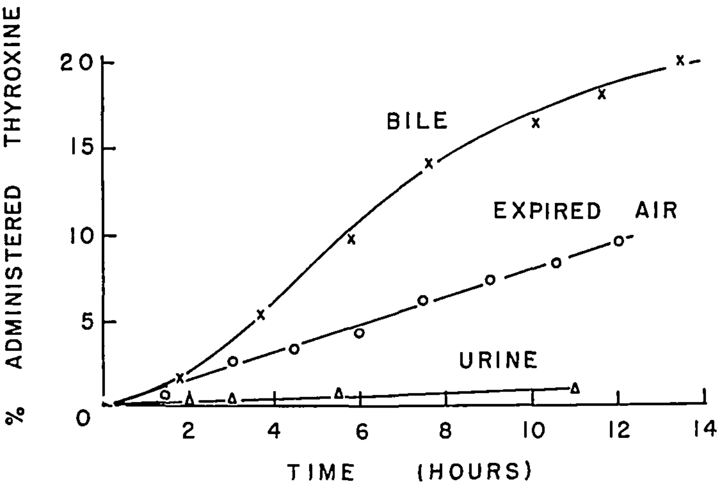

FIG. 1. Elimination of C^{14}-carboxyl labeled thyroxine by different routes.

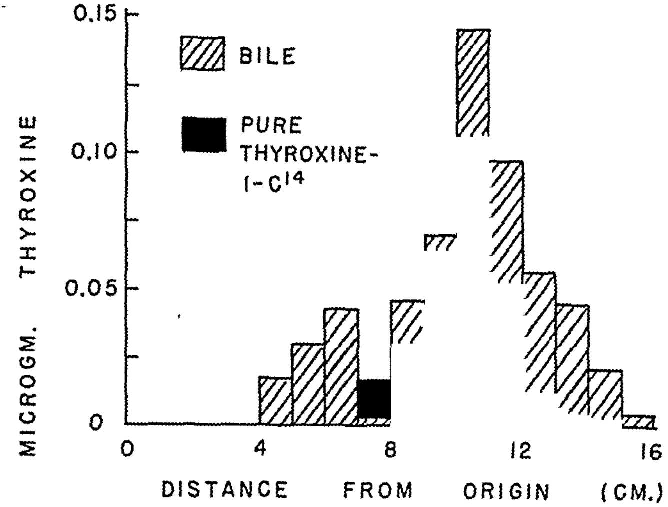

FIG. 2. Chromatogram of 0.1 ml. aliquot of a 2 ml. sample of bile, taken 1.8 to 3.8 hours after administering thyroxine-1-C^{14}.

olized. Essentially the same rate of elimination of $C^{14}O_2$ was obtained in 5 other experiments.

A relatively low C^{14} concentration, about 1% of the total dose, appeared in the urine in 12 hours. The average value was 1.2% for 10 such experiments. This radioactivity may represent, in part, urea derived from $C^{14}O_2$ by way of the ornithine cycle. Gross and Leblond (1949) reported a urinary excretion of about 10% of intravenously administered I^{131}-thyroxine in rats after 24 hours. Most of the I^{131} could not be extracted from the urine by butanol, and was referred to as "non-thyroxine" iodine.

The results of a paper chromatogram performed on bile from a rat given C^{14}-thyroxine is shown in Figure 2. The small peak accounted for 10%, and the larger, more rapidly moving peak 53%, of the total C^{14} in the bile sample. The position of the larger peak is identical with that of thyroxine. These results show that, at the 780 μgm./kilo dosage, free thyroxine predominates in the bile. This conclusion is in accord with the finding of Taurog and co-workers (1952) that large doses of thyroxine-I^{131} are conjugated to only a very limited extent in the rat. Because of the limitations imposed by the specific radioactivity of our thyroxine-1-C^{14}, it was not possible to employ the latter at physiological levels.

The data in Figure 3 indicate that virtually all of the C^{14} of the bile is released by ninhydrin treatment. The agreements are within the limits of experimental error. Van Slyke, Dillon, MacFadyen, and Hamilton (1941) have shown that amino acids are decarboxylated by ninhydrin only when both amino and carboxyl groups are in the uncombined state. Although

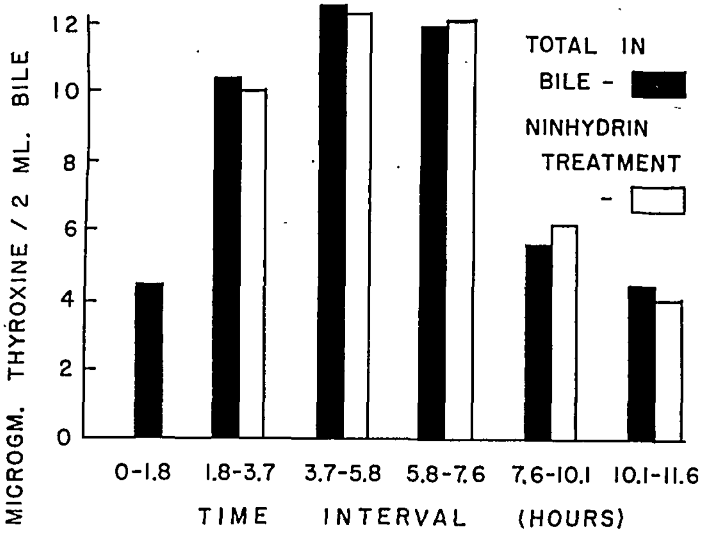

FIG. 3. Thyroxine excretion, as measured by total C^{14} in bile, and by $C^{14}O_2$ released from bile by ninhydrin.

chromatography indicates that a minor portion of the thyroxine exists in conjugated form, this fraction may be bound through the phenolic hydroxyl group, and hence would still react with ninhydrin.

SUMMARY

The various routes of elimination of thyroxine-1-C^{14} in normal adult rats were investigated. Labeled thyroxine was administered subcutaneously at a level of 780 μgm./kilo. The bile was the major route of excretion, and contained about 20% of the C^{14} after 12 hours. Approximately 10% of the C^{14} appeared in the expired CO_2, and 1% in the urine, in this period.

Paper chromatographic analysis of a bile sample taken between 2 and 4 hours, revealed the presence of a small secondary component, in addition to a large thyroxine peak. Treatment of bile samples with ninhydrin resulted in a quantitative release of C^{14} as $C^{14}O_2$. This finding indicates that both amino and carboxyl groups of the thyroxine in the bile are in an uncombined state.

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ESTIMATED DURATION OF THE SPONTANEOUS ACTIVATION WHICH CAUSES RELEASE OF OVULATING HORMONE FROM THE RAT HYPOPHYSIS[1]

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INTRODUCTION

NEUROGENIC activation of the rabbit adenohypophysis causing discharge of ovulating hormone is now considered to be accomplished within about one minute after coitus (Sawyer, Markee *et al.*, 1947, 1949). Actual discharge into the bloodstream of hormone sufficient for ovulation requires approximately one hour in that species (Fee and Parkes, 1929).

Whereas, in spontaneously ovulating animals, discharge of the hormone was long thought to be invoked by direct action of ovarian steroids on the hypophysis, there is now strong evidence that this effect is mediated by the hypothalamus (Sawyer, Everett and Markee, 1949; Everett and Sawyer, 1949). Activation of the hypophysis in cyclic rats occurs within a restricted period of time during the day of proestrus (Everett, Sawyer and Markee, 1949). In our colony this critical period is between 2 and 4 P.M. for the great majority of animals (4-day cycle), about 9 to 11 hours before ovulation. The injection of certain blocking agents at 2 P.M. or earlier will prevent ovulation, while their injection at 4 P.M. will not usually interfere with it.

Studies with the rabbit have demonstrated that antiadrenergic agents of the β-haloalkylamine series (Dibenamine and SKF-501) and certain anticholinergic agents (atropine and Banthine) will block the reflexogenous stimulus to the hypophysis when injected intravenously within a fraction of a minute after coitus (Sawyer, Markee *et al.*, 1947, 1949, 1950, 1951). These agents are similarly effective in blocking the "spontaneous" activation of the hypophysis in cyclic rats. In addition, the barbiturates have been effective in the latter species, apparently because they can be intro-

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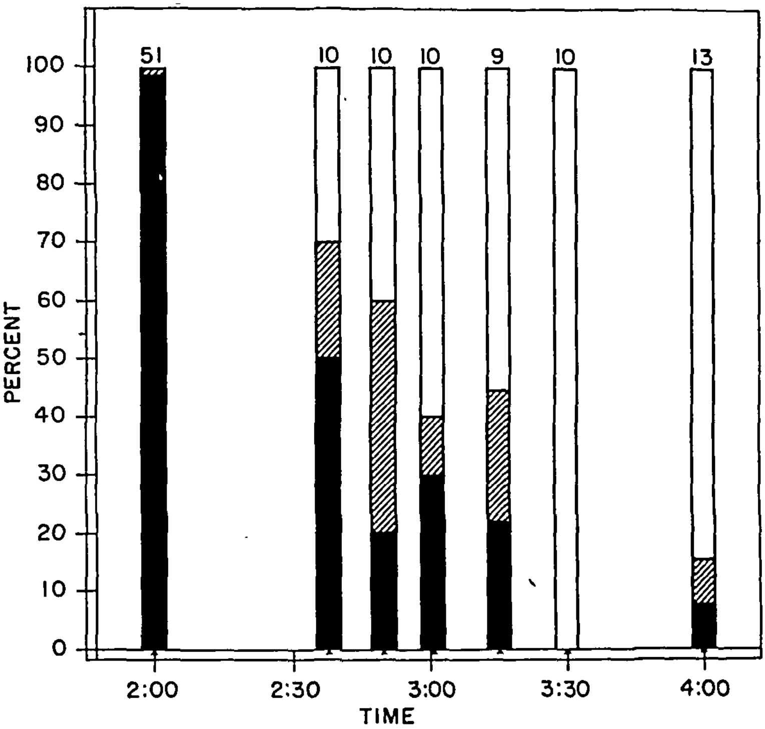

FIG. 1. Results of injection of atropine on the day of proestrus at different times between 2:00 and 4:00 P.M. Solid black: complete blockade. Cross-hatching: partial blockade. White: complete ovulation. The number above each bar indicates the respective number of rats in that time-group. The 2:00 and 4:00 P.M. data are from previous work with atropine and Nembutal (From Everett, 1952, by courtesy of the Ciba Foundation).

gression, for the following considerations. During the first 50 minutes 65% of 20 rats had begun activation, while during the succeeding 40 minutes the percentage increased to only 83% of the remaining 29 rats. Even as late as 4 P.M. 1 case of complete blockade and 1 of partial blockade were encountered. While the former may have been a rare animal which whould have failed to ovulate without atropine, the latter was not. In her case activation must have begun only a short time before injection. It seems probable that an average of the combined 3:30 and 4 P.M. data gives a more nearly true value than either alone.

The occurrence of 9 cases of partial activation, rather uniformly distributed among the 2:40–3:15 time-groups, confirms the earlier impression that the activation stimulus is not momentary. Such a conclusion, however, requires that under the conditions of administration of atropine in massive doses by the subcutaneous route the drug abruptly assumes full blocking concentration at its locus of action, with no significantly prolonged interval during which it would only moderately diminish the strength of stimulus. The following control experiments seem to give this assurance.

A threshold dose for subcutaneous injection was arrived at empirically: 230 mg./kg. (23 mg. atropine sulphate per ml. of physiological saline). This amount was administered to each of 15 proestrous 4-day cyclic rats at 2:00 P.M. At autopsy next morning the tubes were searched for ova and the excised ovaries were critically examined under the dissecting microscope. In the absence of tubal ova all follicles showing any degree of hyperemia were dissected out with iridectomy scissors and were individually crushed in a small drop of saline between slide and coverslip for microscopic examination. Eight of the 15 rats were judged to have been completely blocked, while 6 rats had ovulated completely. In the remaining animal 3 follicles were markedly hyperemic and when these were crushed prominent corona radiata were seen. Maturation of the ova was in progress and the first polar body was seen in one instance. Partial activation in this one exceptional animal may be assigned to an unusually early beginning of hypophyseal activation, similar to the one case from the Nembutal series included in the 2:00 P.M. group in figure 1. The absence of *numerous* examples of partial activation in the present series, intergrading between complete blockade and complete ovulation, attests an essentially all-or-none effect of atropine at threshold level and signifies that no prolonged interval of partial effectiveness of atropine precedes the time at which full blockade is accomplished.

The second series of control experiments approached the question in another way. If the cases of partial blockade in the definitive series (Fig. 1) were mainly representative of a prolonged interval of partial effectiveness of atropine associated with slow absorption from the subcutaneous site, the intravenous injection of the maximum amount tolerated, immediately before the standard subcutaneous dose at some time during the critical period, should have an all-or-none effect. Most female rats of this strain will tolerate intravenous injection of 70 mg./kg. of atropine sulphate (35 mg./ml. in Ringer-Locke solution). This amount was now given to each of 10 proestrous 4-day cyclic rats at one or another time between 2:30 and 3:20 P.M., the entire amount being introduced within 70 to 80 seconds. (Two additional rats died within a few minutes after injection.) Subcutaneous injection of the 700 mg./kg. dose was completed within a minute afterward. Marked tremors were noted before the intravenous injections were complete and involuntary contractions of various muscle groups were regularly seen when the animal was released. It is significant that in 3 of the 10 rats partial blockade was encountered. At autopsy on the morning after injection, 2 of these had a single follicle showing corona formation and maturation changes, while the third animal had shed 2 ova and most of her unruptured follicles were markedly hyperemic. This considerable number of animals showing partial effects supports the interpretation that they arise from abrupt interruption of a lengthy process of hypophyseal activation.

Inasmuch as the chance of interrupting a progressive process is a direct function of its duration, the latter can be estimated in the present instance from the percentage partially activated. If regression of percentage blocked were linear with respect to time, a direct transformation could be made from percentage partially activated to minutes elapsed. Inasmuch as the regression is curvilinear, however, certain approximations are required to allow such transformation.

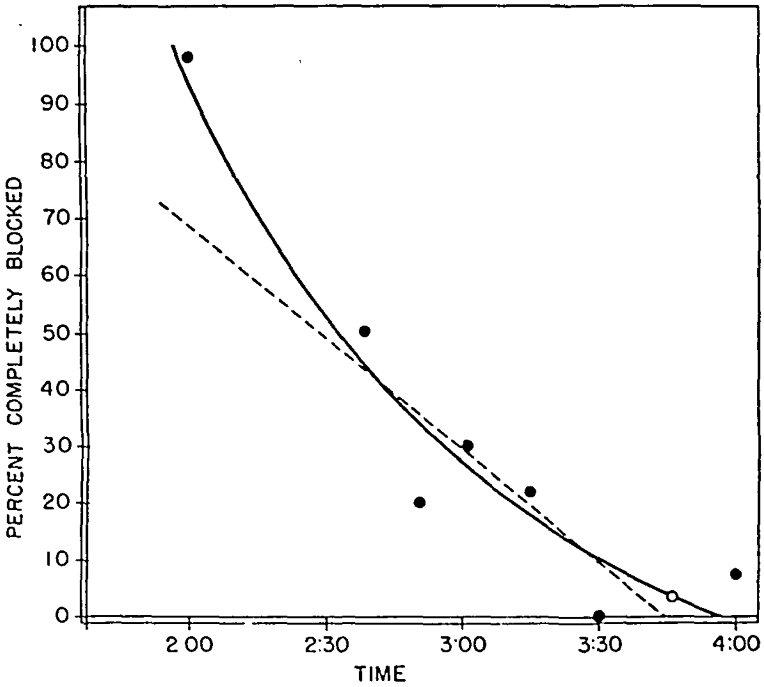

FIG. 2. Diminishing frequency of complete blockade after 2:00 P.M., inversely measuring the beginning of hypophyseal activation.The logarithmic curve is mathematically fitted to the percentages represented by solid circles (the open circle represents the average of the 3:30 and 4:00 P.M. values). The straight (broken) line is approximated to the curve between the 2:40 and 3:30 points (see text).

In Figure 2 a logarithmic curve is mathematically fitted to the points representing percentages completely blocked. A straight (broken) line is approximated to the logarithmic curve between the 2:40 and 3:30 points. The slope of this line is such that 10% on the y axis corresponds to 15.3 minutes on the x axis. In terms of this slope, the 9 cases (18.4%) of partial activation among 49 rats of the 2:40–3:30 groups correspond to 28 minutes on the time axis.

This value, however, is admittedly a very rough approximation and has little significance in itself. An important source of error is chance variation in the percentage partially activated. By use of Fisher's table VIII$_1$ (Fisher and Yates, 1949), entered with $a = 9$ ($a/N = 9/49 = 0.184$) the lower limits of expectation were obtained.[3] At probability levels of 0.025 and 0.005 these limits were 10% and 8%, respectively, corresponding to 13 and 10 minutes (fig. 3). Thus, the probability is high (0.975) that duration of stimulation is greater than 13 minutes, based on the slope of the straight line from figure 2. An extremely unlikely lower limit may be calculated by disregarding the evident curvilinearity of regression and employing as the steepest plausible slope a straight line drawn between the 2:40 P.M. and 3:30 P.M. values. Since on this assumption all activation would be completed before 3:30, this group is excluded in calculating the percentage partially activated and Fisher's table VIII$_1$ is entered with $a = 9$ ($a/N = 9/39 = 0.231$). At the probability level of 0.005 this gives a lower limit of 9 minutes (Fig. 3, lower dotted line).

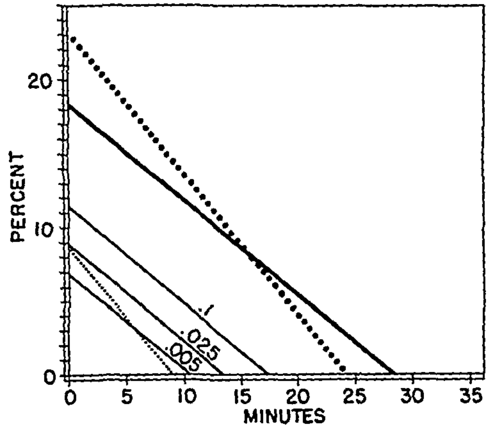

FIG. 3. Relation between percentage partially blocked and time. The heavy solid line has the same slope as the broken line in figure 2. Lower limits of expectation at three levels of probability are represented by the narrow solid lines. Significance of the *dotted lines is given in the text.*

The calculated limits do not take into consideration the numerous cases of partial activation in other comparable experiments, e.g. the progesterone-atropine series cited earlier (Everett and Sawyer, 1949). Among 78 appropriate examples from such experiments, 20 (24%) were classed as partial activation. Thus the frequency of partial activation in the present series appears to be more truly representative than the calculations imply. Consequently it seems reasonable to judge that in any given rat the duration of stimulus is within the range of 20 to 35 minutes.

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[3] The upper limit was given directly by the fact that 3 of 10 rats in the 2:40 group were fully activated. Their periods of activation must have been less than 40 minutes.

DISCUSSION

The results confirm the earlier conclusion (Everett, Sawyer and Markee, 1949; Everett and Sawyer, 1950) that in 4-day cyclic rats of our colony a specific neurogenic stimulus, essential to the discharge of ovulating hormone, passes to the adenohypophysis within restricted, predictable time limits on the afternoon of the day of proestrus. In addition, it is now shown that this stimulus in any one individual is considerably shorter than the 2-hour critical period (2 to 4 P.M.) previously defined. On the other hand, the stimulus lasts much longer than the reflexogenous one in the rabbit. In that species the cholinergic (atropine-sensitive) phase is thought to be completed early in the first minute post-coitum, while the adrenergic (Dibenamine-sensitive) phase is only slightly longer, approximately 1 minute (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949; Sawyer, Markee and Everett, 1950). In the rat the cholinergic phase of the stimulation process lasts for over 9 minutes, more probably between 20 and 35 minutes and certainly less than 40 minutes.

It seems likely that in the rat the central (Nembutal-sensitive) phase and the cholinergic and adrenergic phases of the stimulation process take place almost concurrently. Time differences of the order found in rabbits would be obscured by the far greater over-all duration.

A quantitative relationship apparently exists between length of time of hypophyseal stimulation and the amount of ovulating hormone released. Such an interpretation rests on the finding in rabbits that none of the blocking agents interferes directly with passage of ovulating hormone from the hypophyseal cells into circulation (Sawyer, *et al.*, loc. cit.). Since a species difference in this respect is unlikely, the numerous cases of partial blockade in the present series should then represent incomplete release resulting from foreshortened stimulation. Indeed, if the normal period of stimulation approximates half an hour, stimulation and release may proceed concurrently. While in rabbits about an hour is required for release (Fee and Parkes, 1929), the time in rats could easily be somewhat shorter.

It should be noted that in at least two of the species in which ovulation depends on copulation, relatively prolonged stimulation from lengthy or repeated copulation is indicated (*ferret,* Hill and Parkes, 1932; *mole shrew,* Pearson, 1944). In the mole shrew 1 to 3 copulations per day are said to be ineffective, whereas ovulation regularly follows when the number is increased to 10 to 15 for another two days. In contrasting the rabbit and the ferret, Hill and Parkes spoke of the "trigger" mechanism characteristic of the former species, although its momentary nature had not been elucidated at that time. It seems probable that in spontaneous ovulators and reflex ovulators alike there may be rather wide species variation in the duration of the stimulation process.

There are some indications that under normal circumstances the rat hypophysis continues to be stimulated for a considerably longer period than that which is just sufficient to release the minimal amount of gonadotrophin capable of ovulating all the follicles. Everett, Sawyer and Markee (1949) noted in the original 4 P.M.-atropine series that while all animals ovulated, the corpora lutea from the preceding cycle failed to store visible cholesterol within the usual time. Such cholesterol storage has been shown to be a function of LH (Everett, 1947). Another function of LH is seen in the depletion of cholesterol in the interstitial tissue (Claesson and Hillarp, 1947; Everett, 1949). In our 4-day cyclic rats such depletion is normally so extreme on the morning after ovulation that the ovarian interstitium presents a dull, watery appearance. In the present experiments this extreme depletion was noted in only 24 of 35 rats (69%) in the "fully ovulated" category. It seems likely, therefore, that the estimate of 20 to 35 minutes simply represents the minimal stimulation necessary for ovulation and that the normal stimulation lasts for some longer time, causing relase of an excess of gonadotrophin. This factor of insurance may be reflected not only in the lipid changes in corpora lutea and interstitial tissue, but also in the uniformity of ovulation time observed in these animals.

SUMMARY

Previous study of 4-day cyclic rats (inbred Vanderbilt strain) has defined a 2-hour interval during proestrus, within which neurogenic ovulatory activation of the adenohypophysis takes place. In the present study, 49 such rats were injected with the standard blocking dose of atropine at different times during that interval and were autopsied the next morning. From the results, in particular from the relative frequency of partial blockade, it is estimated that to release sufficient gonadotrophin for complete ovulation the required duration of stimulus is between 20 and 35 minutes. Collateral evidence suggests that the stimulus may normally continue somewhat longer, serving to release an excess of ovulating hormone.

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ORGANIC PHOSPHORUS COMPOUNDS IN THE TESTIS OF THE RAT AT VARIOUS AGES[1,2]

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NUMEROUS investigations have been made of the pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) concentrations of various tissues and organs in different experimental animals. These have been summarized in tabular form by Davidson (1947, 1950), Schneider (1946) and Schneider and Klug (1946).

Cytochemical studies covering the content and distribution of nucleic acids in many tissues and organs have been reviewed by Davidson (1950), Brachet (1950) and Caspersson (1950).

Examination of the literature has shown that little attention has been given to the concentration and distribution of these important substances in the testis. McCarter and Stelges (1948) in an examination of methods for the determination of phosphorus-containing constituents in rat tissues have included a limited survey of organic phosphorus compounds in the testis. Using cytochemical techniques Brachet (1933, 1940, 1941) has studied insect and amphibian testes and Caspersson (1933, 1936) grasshopper testes.

The paucity of information concerning the nucleic acids of the testis, particularly the mammalian testis, has suggested the desirability of such a study in the rat by chemical and cytochemical techniques. In order to provide a more complete survey of other phosphorus-containing substances additional studies were made.

Acid-soluble phosphorus (ASP) which according to Lipman (1941) and

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Kalcar (1941) represents primarily intermediates and enzymes in carbohydrate and energy metabolism, was determined because of the reputed relationships between these and nucleic acid metabolism (Hevesy, 1948; Davidson, 1950). The lipid phosphorus (LP) fraction was studied since Claude (1939, 1940, 1941, 1949) has demonstrated a relationship between cytoplasmic pentose nucleic acid and phospholipid.

The present report, therefore, includes chemical data on the four phosphorus fractions and cytochemical data on the nucleic acids in the rat testis at different ages. Similar data for testes with experimentally induced impairments in spermatogenesis are being reported in a separate paper (McEnery and Nelson, 1952).

MATERIALS AND METHODS

Rats of the Long-Evans strain raised in this laboratory were used throughout the study. Animals were sacrificed when ten, twenty, thirty, forty, sixty and one hundred eighty days of age and the testes removed for chemical analysis and for cytochemical and routine histological preparations.

Chemical Determinations

Pentose nucleic acid phosphorus (PNAP), desoxypentose nucleic acid phosphorus (DNAP), total nucleic acid phosphorus (TNAP), acid soluble phosphorus (ASP) and lipid phosphorus (LP) were determined according to the method of Schmidt and Thannhauser (1945) as modified by Schneider (1946). In the case of each procedure duplicate determinations were made on testicular tissue from each animal except in the ten day series where the two testes from each of five animals were pooled. All were wet weight determinations and moisture contents were checked in each group.

Cytochemical Preparations

Portions of the testes were fixed in Zenker acetic fluid for one hour and fifteen minutes and prepared according to the Feulgen technique (Feulgen and Rossenbeck, 1924) for DNA and the methyl green pyronin technique for PNA and DNA (Brachet 1940, 1941). Since the latter technique is extremely capricious and for specificity should be accompanied by ribonuclease digestion of control sections the cytoplasmic basophilia demonstrated by it was used only as a rough index of PNA. It may be mentioned, however, that Taft (1951) in a recent paper offers substantial evidence for the specificity of the methyl green pyronin reaction as an indicator of the presence of both PNA and DNA.

Cytological Preparations

Portions of the testes were fixed in Bouin's fluid and stained with hematoxylin and eosin, Masson's and Mallory-Azan procedures.

RESULTS

Chemical Determinations

Table 1 presents the mean values for the concentrations of phosphorus in the PNA, DNA, TNA, ASP and LP fractions, and the calculated amounts of PNA and DNA along with standard errors and p values of the differences between the means of the values obtained for these fractions in the animals of the younger age groups as compared with the equivalent values in adult animals. The corresponding absolute values (calculated amounts in both testes) are given in Table 2.

It is readily seen that as the age of the animals progresses toward maturity the testicular concentration of each of the phosphorus fractions decreases with the exception of the lipid fraction which remains approximately constant (Fig. 1). Conversely in terms of absolute amounts there is a progressive increase in each of the phosphorus fractions with increase in age (Fig. 2).[4]

TABLE 1. THE MEAN CONCENTRATIONS OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN CONCENTRATION OF PNA AND DNA FOR EACH AGE EXPRESSED AS MG. PER 100 GM. OF FRESH TISSUE

Group	No. of rats	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
10 day	24	120.01 ±6.459* 0.001†	25.81 ±0.964 0.001	141.32 +2.632 0.001	148.79 ±2.454 0.001	34.13 ±1.718 0.20	1272.10	260.68
20 day	22	110.84 ±0.417 0.001	26.67 ±0.842 0.001	137.85 ±2.213 0.001	126.98 ±1.707 0.001	37.39 ±0.861 0.01	1174.90	269.36
30 day	21	86.63 ±1.546 0.001	20.83 ±0.562 0.001	107.35 ±1.765 0.001	94.72 ±2.038 0.001	32.70 ±2.071 0.20	918.27	210.38
40 day	22	70.31 ±1.997 0.001	13.86 ±0.521 0.001	86.60 ±1.675 0.001	73.55 ±2.115 0.001	33.76 ±1.191 0.20	748.28	139.98
60 day	20	59.45 ±1.794 0.20	12.21 ±2.552 0.20	73.94 ±1.862 0.20	67.67 ±2.119 0.20	34.76 ±1.113 0.20	630.17	123.32
Normal Adult (180 day)	27	60.32 ±0.639	12.30 ±0.215	75.00 ±1.002	65.33 ±1.023	32.72 ±1.065	639.39	124.23

* Standard error of the mean.

† p values (from students table of t, Fischér and Yates 1947) for the differences between the mean values of the different age groups and the mean values of the normal adult group.

Total phosphorus determinations were made on five samples from normal adult testes and the mean values of these were compared with the sums of the mean values for the four fractions in the same testes. The total phosphorus content was greater by 21.73 mg. per 100 gm. and this difference presumably represents phosphoprotein (Schmidt and Thannhauser, 1945; McCarter and Stelges, 1948) and, of course, experimental error.

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[4] Values for individual analyses and numerous illustrations may be found in the Ph.D. dissertation of the senior author (McEnery, 1952).

TABLE 2. THE CALCULATED MEAN VALUES OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN VALUES FOR PNA AND DNA FOR EACH AGE EXPRESSED AS MG. IN BOTH TESTES

Group	No. of rats	Wt. of testes in gm.	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
10 day	24	0.041	0.049 ±0.002* 0.001†	0.010 ±0.0004 0.001	0.058 ±0.002 0.001	0.061 ±0.002 0.001	0.014 ±0.0006 0.001	0.527	0.108
20 day	22	0.209	0.231 ±0.006 0.001	0.055 ±0.001 0.001	0.0288 ±0.010 0.001	0.265 ±0.008 0.001	0.078 ±0.002 0.001	2.453	0.562
30 days	21	0.578	0.501 ±0.014 0.001	0.120 ±0.003 0.001	0.623 ±0.014 0.001	0.544 ±0.013 0.001	0.193 ±0.006 0.001	5.318	1.214
40 day	22	1.724	1.214 ±0.045 0.001	0.240 ±0.010 0.001	1.432 ±0.037 0.001	1.275 ±0.041 0.001	0.585 ±0.023 0.001	12.873	2.432
60 day	20	2.520	1.475 ±0.056 0.001	0.304 ±0.012 0.010	1.838 ±0.068 0.001	1.568 ±0.006 0.001	0.864 ±0.036 0.010	15.641	3.079
Normal Adult (180 day)	27	3.100	1.901 −0.042	0.387 −0.009	2.366 −0.052	2.053 −0.043	1.024 −0.029	20.155	3.913

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different age groups and the mean values of the normal adult group.

Cytological and Cytochemical Observations

Normal Adult (One hundred and eighty days). The histologic and cytologic pictures are well known and need not be detailed here. Reference to them will be made in the course of the discussion since the changes in some of the phosphorus fractions are explained best in terms of changes in the cellular population of the testis.

The Feulgen preparations showed that the nuclei of the fibroblasts, endothelial cells and mast cells which are present in the intertubular areas are rich in fine granules of DNA while those of the Leydig cells contain only a few coarse granules. The sperm heads are Feulgen-positive throughout, and the nuclei of spermatocytes and spermatogonia give a strong positive reaction. The nuclei of the spermatids are strikingly low in DNA and the same is true for the Sertoli cell nuclei.

The methyl green pyronin preparations indicate the presence of considerable basophilia in the cytoplasm of the Leydig cells and much smaller amounts in other intertubular cells. All of the tubular cells possess cytoplasmic basophilia, the amount being proportional to the amount of cytoplasm in the cells. The Sertoli cells and spermatogonia appear to contain a little more than the others, and in the area where sperm maturation is

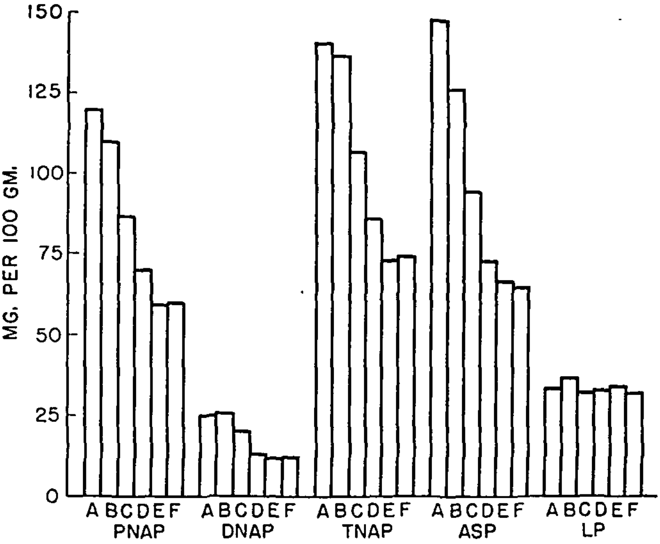

FIG. 1. Graph of the mean concentrations of the five P fractions for each age.

A = 10-day
C = 30-day
E = 60-day
B = 20-day
D = 40-day
F = 180-day

PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

taking place there are numerous extracellular, red, basophilic droplets. These droplets are not seen in all tubules but apparently coincide with a phase in the spermatogenic wave.

Ten-Day-Old Series. The testis of a ten-day-old rat contains occasional intertubular elements, which appear to be somewhat differentiated in the direction of Leydig cells. However, most of the cells in these areas are various types of connective tissue elements. The tubules contain Sertoli cells, actively dividing spermatogonia, and possibly a few young spermatocytes.

The intertubular cell nuclei give a Feulgen reaction comparable to that seen in the cells of these areas in the adult testis. Much the same is true of the nuclei of the tubular cells; indeed, the reaction for the Sertoli cells is somewhat more marked than in the Sertoli cells of the adult testis.

Those cells in the intertubular areas which appear somewhat differentiated are high in cytoplasmic basophilia while the mesenchymal cells give a weak reaction since they contain very little cytoplasm. In all tubular cells the cytoplasm is strongly basophilic.

Twenty-Day-Old Series. There are no Leydig cells in the testis of a twenty-day-old rat, the intertubular areas being made up of connective

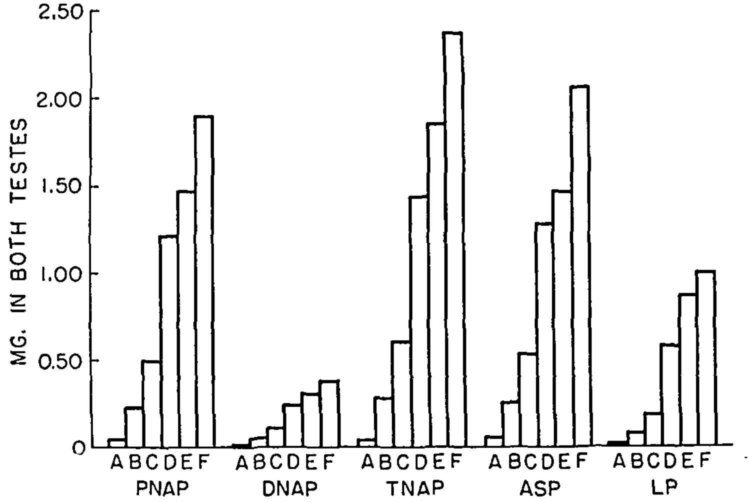

FIG. 2. Graph of the mean absolute values of the five P fractions for each age.

A = 10-day	C = 30-day	E = 60-day
B = 20-day	D = 40-day	F = 180-day

PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

tissue with a few mast cells, capillaries and other small blood vessels. The tubules contain Sertoli cells along with spermatogonia and spermatocytes.

The nuclei of all intertubular cells give a positive Feulgen reaction of about the same intensity as in similar cells of the adult testis. The Sertoli cell nuclei show a characteristic light diffuse pattern and the spermatogonia and spermatocyte nuclei both are rich in Feulgen positive material.

As in the ten-day-old testis the mesenchymal elements have little cytoplasm and show very little basophilia. The cytoplasm of the Sertoli cells, spermatogonia and spermatocytes is more basophilic than that of like cells in the adult testis.

Thirty-Day-Old Series. The testis of a thirty-day-old rat contains a few Leydig cells and the tubules have Sertoli cells, spermatogonia, spermatocytes and some spermatids.

The nuclei of the cellular elements of both the tubular cells and the intertubular areas give a Feulgen reaction similar to that seen in the normal adult. The Leydig cells have only a small amount of Feulgen-positive material and the spermatids give a weak reaction.

In methyl green pyronin preparations the Leydig cells show cytoplasmic basophila as do the Sertoli cells, spermatogonia, spermatocytes and spermatids. The Sertoli cells and spermatogonia appear to stain a little more deeply than the spermatocytes and spermatids.

Forty-Day-Old Series. With the appearance of some sperm the forty-day-old testis assumes adult characteristics and the Feulgen picture is almost identical with that seen in normal adult testes. The amount of cytoplasmic basophilia in the testes of animals of this series appears to be in general a little greater than that present in adult testes.

Sixty-Day-Old Series. The testes of sixty-day-old rats are identical cytologically and cytochemically with those of normal adult rats.

DISCUSSION

The observations on the normal adult rat testis which have been made in this investigation confirm, in most respects, such information as is available. Chemical data for all fractions are essentially in agreement with those reported by McCarter and Stelges (1948) with the exception of the LP fraction which they found to be 59 mg. per 100 gm. of tissue as compared with our value of 32.72 mg. per 100 gm. of tissue. The discrepancy may be due in part at least to the fact that we did not use the extensive extraction procedure which they employed. However, in our experience and in that of Schneider (1946), such a laborious fractionation did not materially increase the yield of LP. It is quite possible that on the basis of a limited number of analyses such as they carried out an inconsistently high value could easily be obtained.

The cytochemical observations on the normal adult testis which have been made in this study are entirely consistent with those reported previously. Brachet (1933, 1940, 1941) has shown that in insects and amphibians the spermatogonia contain more pentose nucleic acid than the other germ cells. In our preparations we noted more cytoplasmic basophilia in the peripheral cells of the tubules; however, it was somewhat difficult to decide whether or not this greater amount was due entirely to spermatogonia or whether the Sertoli cells were also involved. Caspersson (1933, 1936) working with grasshopper testes has shown that the amount of PNA is greatly increased in the older spermatocytes. It was our experience that the spermatocytic as well as the spermatogonial nuclei give a strong Feulgen positive reaction. This is in agreement with the theses of Brachet and Caspersson that the synthesis of DNA occurs at this time and that it is dependent on the amount of PNA present in spermatogonia and primary spermatocytes. We also found that the spermatid nuclei stained very lightly with the Feulgen reagent, an observation which is easily reconciled when we consider that the spermatid nuclei contain a haploid number of chromosomes. The occurrence of large basophilic droplets in the region of sperm maturation is particularly striking in view of evidence presented by McEnery and Nelson (1950, 1951) that there is accumulation of sudanophilic and Schiff positive lipid in the same distribution. Two possible explanations may be offered for this finding. Lipoprotein complexes in the blood are well known (Troensgaard and Koudahl, 1926; Machebeouf, 1929; Machebeouf and Sandar, 1932; and Turner and Gibson, 1932) and it would seem possible that such a complex arising as a nutritive substance from the Sertoli cells, whose function is believed to be sustentacular and nutritive (Elftman, 1950; Nelson, 1951), would be visualized by lipid procedures as well as those for nucleoprotein. The second and possibly more reasonable explanation, in view of the fact that the appearance of these droplets coincides with a phase in the spermiogenic wave (Roosen-Runge, 1950, 1951), is that they arise from the sloughed cytoplasm of the spermatid during its metamorphosis. The latter explanation would be in good agreement with the observations of Claude (1939, 1940, 1941, 1949) that pentose nucleic acid exists in the cytoplasm in combination with phospholipid. Thus it is possible that the remnants of such a pentose nucleic acid-phospholipid complex are released when the spermatid is transformed into a spermatozoon and are visualized by the respective cytochemical procedures.

During the attainment of the adult condition changes in the different phosphorus fractions follow rather uniform patterns and the cytochemical observations are in good agreement with the patterns for the concentration of the nucleic acids. The concentrations of all fractions, with the exception of LP which remains relatively constant, decrease progressively as the age of the testis increases (Fig. 1), while the absolute values (amounts in both testes) for all of the phosphorus fractions increase progressively with the age of the testis (Fig. 2).

Adequate interpretation of these findings requires that we examine the morphologic changes which take place in the testis between ten and one hundred eighty days of age. In the first place there is approximately an eighty fold increase in weight. This is brought about by increases in the size of cells and by increases in their number. Secondly, the attainment of maturity involves the appearance of new cell types. The former, as we shall point out, appears to exert the major influence on the absolute values of the phosphorus fractions while the latter exerts more influence on the variations in concentration.

Two points must be developed in order to clarify this. In the first place it is well known that PNA is found chiefly in the cytoplasm. This is apparently also the case for the ASP fraction. Therefore tissue made up of cells having a large amount of cytoplasm (low nuclear-cytoplasmic ratio) will be high in those two substances whereas tissue made up of cells having little cytoplasm (high nuclear-cytoplasmic ratio) will have low concentrations of these substances. DNA on the other hand is for the most part (probably exclusively) in the nucleus and is actually a very important constituent of the chromosomes. Thus the DNA content is affected by the nuclear cytoplasmic ratio of cells making up a given tissue. Furthermore, in the case of the testis there are cells with haploid as well as cells with diploid numbers of chromosomes. This will also influence the content of DNA. In fact, Boivin, Vendrely and Vendrely (1948) found that the nuclei of somatic cells from animals of a given species have twice as much DNA as the corresponding sperm (haploid) nuclei. Thus in the adult testis the concentrations of the various phosphorus fractions will be a function of the numbers of the various cell types present at that time.

In the second place attention must be directed to the cytologic picture of the testis at each age included in this study. At ten and twenty days of age the seminiferous tubules of the rat testis contain only cells which have considerable cytoplasm, namely Sertoli cells, spermatogonia and primary spermatocytes, while at thirty days secondary spermatocytes and spermatids have made their appearance and at forty days sperm are in evidence. After forty days no new cell types are added and further changes involve only increases in the numbers of cells, particularly those of the more mature types. Three significant generalizations can therefore be made with regard to the growth and maturation of the testis. First, as new cell types are added the total nuclear-cytoplasmic ratio of the cells of the testis changes from a lower to a higher value. Secondly, the last three cell types to appear, namely, the secondary spermatocytes, spermatids and sperm, reduce the total number of chromosomes per cell in the testis. Thirdly, the great increase in numbers of these latter cell types influence not only the concentrations by dilution of the cell population but also the absolute values by adding materials to the testis as a whole.

That the PNAP concentration was highest at ten and twenty days is entirely consistent with the cytologic picture. When, however, the cytoplasmic basophilia was examined in these two groups, it was found to be more intense than in corresponding cells in the adult group and further, that it was somewhat greater in the ten than in the twenty day testis. Thus it would seem that testes of young rats have a higher concentration of PNA than can be accounted for entirely on the basis of the cell types which are present. Since slightly elevated values for PNA have been found generally in young growing tissues and greatly increased amounts, as compared to adult tissues, have been observed in embryonic tissues (Davidson, 1950) it is not surprising to find that the PNA values in the testes of young rats, which are similar in some respects to embryonic tissues, are markedly higher than in adult testes. It is worth noting that from the tenth to the twentieth day the testis undergoes a fivefold increase in weight and from the twentieth to the thirtieth day a threefold increase. Therefore, the high concentration can be attributed in part at least to the accelerated rate of growth in young testes.

At thirty and forty days the lower PNA concentrations are entirely

ORGANIC PHOSPHORUS COMPOUNDS IN THE TESTIS OF THE RAT UNDER EXPERIMENTAL CONDITIONS WHICH IMPAIR SPERMATOGENESIS[1,2]

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IN SPITE of the concerted efforts which have been made to clarify the functional position and metabolic significance of nucleic acids very little in the way of established interpretations can be offered. Caspersson (1950) and Brachet (1950) have discussed the significance of nucleic acids in the synthesis of cellular proteins, Claude (1940, 1941, 1942, and 1948) has shown that cytoplasmic pentose nucleic acid (PNA) occurs in combination with phospholipids and Hevesy (1948) and Davidson (1950) have pointed out certain relationships between PNA and the components of the acid-soluble phosphorus (ASP) fraction.

It has been reported that fasting and protein-poor diets (Davidson and Waymouth, 1944; Davidson, 1945), ischemia (Drochmans, 1947), adrenocorticotrophic hormone and high carbohydrate diet (Baker, *et al.*, 1948), and hypophysectomy (Geschwind, Li and Evans, 1950) all cause a decrease in pentose nucleic acid (PNA) but no change in desoxypentose nucleic acid (DNA) in the liver. McShan *et al.* (1950) reported a marked increase in PNA but only a slight increase in DNA in the crop glands of pigeons treated with lactogenic hormone. An increase in both PNA and DNA has been observed in the livers of pregnant rats (Campbell and Kosterlitz, 1947) and Stutz and Verzar (1947) reported that thymectomy did not alter the nucleic acid content of the rat liver. Mitchell (1942, 1943) has observed that x-irradiation of various normal and cancerous human tissues produces an accumulation of PNA in the cytoplasm, and Stowell

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(1945) reported that x-irradiation prevents the synthesis of DNA in cancerous tissue. The studies of Errera (1947) indicate a direct effect of x-irradiation on the chemical properties involved in the rigidity of the nuceloprotein complex *in situ.*

In a previous paper (McEnery and Nelson, 1952) we pointed out the scarcity of chemical and cytochemical observations on nucleic acids and other phosphorus containing constituents in the testis and reported such information for the testes of rats ranging in age from ten to one hundred and eighty days.

This report offers similar observations on the testes of hypophysectomized, estrogen-treated, x-irradiated and cyptorchid rats. The experimental procedures to which these rats were subjected were chosen for their well-known effects on spermatogenesis.

MATERIALS AND METHODS

Rats of the Long-Evans strain raised in this laboratory and of the Sprague-Dawley strain obtained from a commercial breeder were used in this study.

Animals were hypophysectomized[4] at weights varying from 150 to 250 gm. and were sacrificed 30 days post-operatively. All animals were examined for completeness of operation.

In the estrogen-treated animals treatment was initiated when the animals weighed approximately 150 gm. Subcutaneous injections of alpha estradiol,[5] were made in 50 and 100 gamma doses daily for 30 to 45 days.

In the x-ray series animals weighing 200 to 250 gm. were given 600 roentgen units applied in a single dose to the caudal half of the body and were autopsied 30 days later.[6]

In the cryptorchid series animals weighing 200–250 gm. at the time of operation were made cryptorchid by anchoring the testes to the anterior abdominal wall. One group was sacrificed 80 days post-operatively and the other 200 days after operation.

Chemical Determinations

Pentose nucleic acid phosphorus (PNAP), desoxypentose nucleic acid phosphorus (DNAP), total nucleic acid phosphorus (TNAP), acid soluble phosphorus (ASP) and lipid phosphorus (LP) were determined according to the method of Schmidt and Thannhauser (1945) as modified by Schneider (1946). Duplicate determinations were made on testicular tissue from each animal. All were wet weight determinations and moisture contents were checked in each group.

Cytological and Cytochemical Preparations

Portions of the testes were prepared for cytological and cytochemical observation according to the methods described in the first paper of this series (McEnery and Nelson, 1952).

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[4] The hypophysectomized rats were obtained from the Hormone Assay Laboratory of Chicago, Illinois.

[5] We are indebted to Dr. Edward Henderson of the Schering Corporation for the alpha estradiol.

[6] We are indebted to the staff of the Radiation Research Laboratory of the State University of Iowa for invaluable assistance in irradiating these animals.

TABLE 1. THE MEAN CONCENTRATIONS OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN CONCENTRATIONS OF PNA AND DNA FOR EACH EXPERIMENTAL GROUP EXPRESSED AS MG. PER 100 GM. OF FRESH TISSUE

Group	No. of rats	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
Hypophysectomy	23	87.14 ±1.664* 0.001†	23.45 ±0.481 0.001	109.87 ±1.865 0.001	81.73 ±1.857 0.001	34.47 ±1.010 0.20	923.68	236.84
Estrogen	21	86.01 ±1.993 0.001	23.24 ±0.576 0.001	110.53 ±1.653 0.001	95.28 ±1.230 0.001	33.44 ±0.512 0.20	911.70	234.72
X-ray	19	67.66 ±1.021 0.001	14.63 ±1.516 0.050	82.25 ±1.646 0.001	81.26 ±2.186 0.001	39.60 ±0.989 0.001	717.19	147.76
Cryptorchid (80-day)	18	66.52 ±2.054 0.010	17.32 ±2.235 0.010	84.04 ±2.015 0.001	78.37 ±2.349 0.001	54.85 ±1.747 0.001	705.11	174.93
Cryptorchid (200-day)	24	67.24 ±1.596 0.001	19.42 ±1.676 0.001	85.75 ±1.300 0.001	65.90 ±1.889 0.20	56.55 ±0.962 0.001	712.74	196.14
Normal Adult (180-day)	27	60.32 ±0.639	12.30 ±0.215	75.00 ±1.002	65.33 ±1.023	32.72 ±1.065	639.29	124.23

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different experimental groups and the mean values of the normal adult group.

RESULTS

Chemical Determinations

Table 1 presents the mean values for the concentration of phosphorus in the PNA, DNA, TNA, AS and L fractions, and the calculated amounts of PNA and DNA along with standard errors and p values of the differences between the means of the values obtained for these fractions in the animals of the various experimental groups as compared with the equivalent values in normal adult animals. The corresponding absolute values (calculated amounts in both testes) are given in Table 2, and the values for concentration and absolute amounts are represented graphically in Figures 1 and 2. It will be noted that the concentrations of the various phosphorus fractions showed varying degrees of increase in the different experimental series as compared to the corresponding values for the normal adult rat. On the other hand the absolute values for all fractions were consistently lower than those observed in the normal adult.[7]

The water content of the testes in the experimental groups did not differ more than five per cent from that observed in testes of normal adult rats.

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[7] Values for individual analyses and numerous illustrations may be found in the Ph.D. dissertation of the senior author (McEnery, 1952).

TABLE 2. THE CALCULATED MEAN VALUES OF THE FIVE P FRACTIONS AND THE CALCULATED MEAN VALUES FOR PNA AND DNA FOR EACH EXPERIMENTAL GROUP EXPRESSED AS MG. IN BOTH TESTES

Group	No. of rats	Wt. of testes in gm.	PNAP	DNAP	TNAP	ASP	LP	PNA	DNA
Hypophysectomy	23	0.625	0.548 +0.020* 0.001†	0.146 ±0.004 0.001	0.690 ±0.024 0.001	0.509 ±0.014 0.001	0.216 ±0.009 0.001	5.809	1.481
Estrogen	21	0.766	0.657 ±0.058 0.001	0.173 ±0.011 0.001	0.835 ±0.063 0.001	0.731 ±0.063 0.001	0.255 ±0.022 0.001	6.965	1.755
X-ray	19	0.694	0.469 ±0.012 0.001	0.101 ±0.010 0.001	0.570 ±0.015 0.001	0.564 ±0.019 0.001	0.276 ±0.012 0.001	4.975	1.025
Cryptorchid (80-day)	18	0.755	0.503 ±0.020 0.001	0.130 ±0.005 0.001	0.635 ±0.022 0.001	0.601 ±0.035 0.001	0.416 ±0.019 0.001	5.331	1.318
Cryptorchid (200-day)	24	0.790	0.531 ±0.016 0.001	0.154 ±0.006 0.001	0.678 ±0.019 0.001	0.523 ±0.021 0.001	0.449 ±0.016 0.001	5.632	1.556
Normal Adult (180-day)	27	3.100	1.901 ±0.042	0.387 ±0.009	2.366 ±0.052	2.053 ±0.043	1.024 ±0.029	20.155	3.913

* Standard error of the mean.

† p values (from students table of t, Fischer and Yates 1947) for the differences between the mean values of the different experimental groups and the mean values of the normal adult group.

Consequently differences seen in the phosphorus fractions were not influenced to any appreciable degree by changes in the moisture content.

Cytological and Cytochemical Observations

The picture in the normal adult testis has been described in a previous paper (McEnery and Nelson 1952).

Hypophysectomized Series. In the testis of the hypophysectomized rat ordinary histologic preparations show that the Leydig cells are atrophic and the tubules contain only Sertoli cells, spermatogonia and spermatocytes.

All of the nuclei of the intertubular elements including the Leydig cells, show a strong Feulgen reaction while the tubules (except for some sloughed spermatocytes which stain deeply) present a picture similar to that seen in the same cells of the normal testis.

While the intertubular cells contain little cytoplasm that which is present is strongly basophilic in methyl green pyronin preparations. The degree of cytoplasmic basophilia in the tubular cells, with the exception of some degenerating spermatocytes which are strongly basophilic, is com-

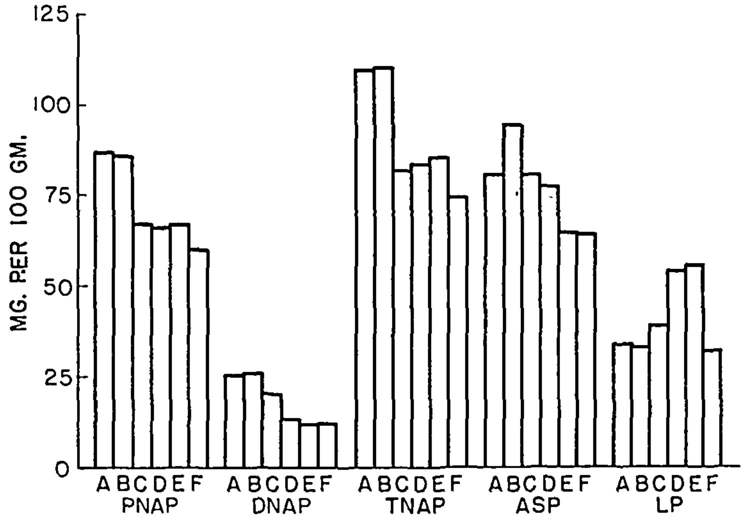

FIG. 1. Graph of the mean concentrations of the five P fractions for each experimental group and for the normal adult group.

A = Hypophysectomy C = X-ray E = Cryptorchid (20-day)
B = Estrogen-treated D = Cryptorchid (80-day) F = Normal Adult
PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

parable to that observed in the same cells of normal testes. In some of the degenerating cells even the nucleus is stained red and occasional red droplets occur free in the tubular lumens.

Estrogen Series. The testes of estrogen treated animals show atrophic Leydig cells and the tubules contain only Sertoli cells, spermatogonia, and spermatocytes. This picture as seen in ordinary histologic preparations is essentially the same as in hypophysectomized animals and is due to the lack of gonadotrophic hormone.

The Feulgen reaction is intense in the nuclei of all intertubular cells. The nuclei of the Sertoli cells contain little Feulgen-positive material while those of the spermatogonia and spermatocytes stain deeply.

The quantity and distribution of cytoplasmic basophilia throughout the testes of these animals appears to be essentially the same as has been described for the testes of hypophysectomized animals. There are, however, few of the degenerating spermatocytes which are so highly basophilic in the latter animals.

X-ray Series. The x-irradiated testes show intertubular areas with an apparent increase in both number and size of the Leydig cells. In x-irradiated testes an extremely variable picture is found in the tubules, some containing only Sertoli cells while others contain sperm, occasional spermatids, a few large basal cells, and some unidentifiable cellular remnants.

The Feulgen reaction in the cell nuclei of the intertubular areas is essentially similar to that seen in the cells of these areas in normal adult testes. Within the tubules the Sertoli cells appear to contain a normal amount of Feulgen-positive material and such other cells as are present give an essentially normal reaction except for the nuclei of the basal cells and cell remnants, both of which stain deeply.

In the methyl green pyronin preparations the cytoplasm of the intertubular cells show approximately the same amount of basophilia as that of corresponding cells in the normal adult testis (a few Leydig and mast cells appear to show more). The residual tubular cells are a little more basophilic than cells of the same types in normal adult testes.

Cryptorchid Series. The testes in both of these groups are essentially similar and will be discussed together. They show the customary apparent

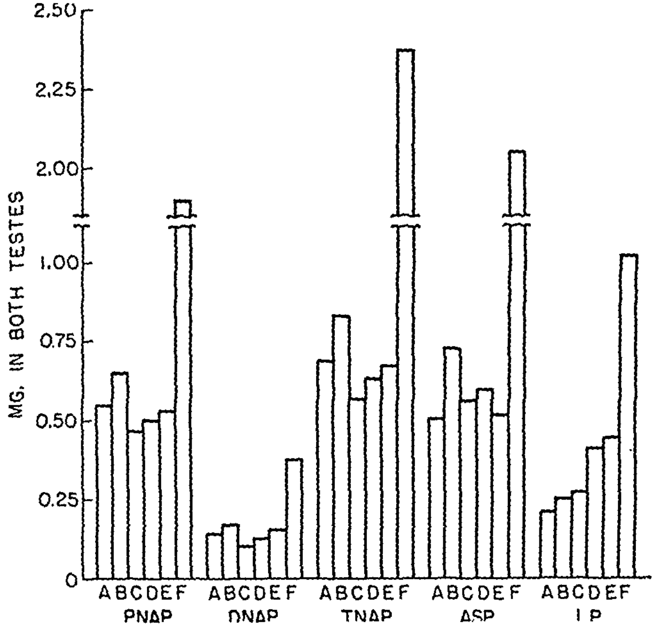

FIG. 2. Graph of the mean absolute values of the five P fractions for each experimental group and for the normal adult group.

A = Hypophysectomy
C = X-ray
E = Cryptorchid (200-day)
B = Estrogen-treated
D = Cryptorchid (80-day)
F = Normal Adult
PNAP = Pentose nucleic acid phosphorus
DNAP = Desoxypentose nucleic acid phosphorus
TNAP = Total nucleic acid phosphorus
ASP = Acid soluble phosphorus
LP = Lipid phosphorus

increase in the number and size of the Leydig cells. The tubules vary with respect to the types of cells present, some having Sertoli cells only and others, in addition, spermatogonia and even occasional spermatocytes.

With the Feulgen stain all of the nuclei of the tubular and intertubular cells show essentially the same reaction as is seen in the corresponding cells of normal adult testes.

The methyl green pyronin preparations reveal an increased amount of cytoplasmic basophilia in the Leydig cells while other intertubular cells show normal quantities. The degree of cytoplasmic basophilia in the tubular cells is also essentially normal.

DISCUSSION

In the first paper of this series (McEnery and Nelson, 1952) the significance of observations on the normal adult testis has been discussed and we have pointed out that variations in the concentration of the phosphorus fractions at various ages was chiefly due to the types of cells which occur in the seminiferous tubules at those ages. The reader is referred to that paper for a detailed discussion of the constancy of position of PNA and DNA in the cell, the relationship of those substances to the amount of cytoplasm and the presence of nuclei with diploid or haploid numbers of chromosomes, and the apparent relationship between the PNAP and ASP fractions. In the LP fractions, on the other hand, such relationships are not evident.

The PNAP concentrations in testes from hypophysectomized and estrogentrated animals showed substantial increases over those found in testes of normal adult rats. Physiologically these two conditions are similar, in that gonadotrophins are absent, and the testicular tubules contain only Sertoli cells, spermatogonia and primary spermatocytes. Furthermore, cytoplasmic basophilia in these cells is no greater than that seen in the normal adult (except for a few disintegrating cells which could not alter the picture significantly). Thus it seems likely that the increased PNAP concentration in these two groups is probably due to the presence of cells with relatively large amounts of cytoplasm rather than to any specific effect of the treatment on PNAP since the cells listed above contain larger amounts of cytoplasm than do secondary spermatocytes, spermatids and spermatozoa.

The testes of x-irradiated animals showed some increase in the concentration of PNAP. These results agree with those of Mitchell (1942, 1943) in spite of the fact that our animals were sacrificed thirty days following treatment while Mitchell examined his tissue within nine hours post-irradiation. The possibility exists that in our study we were dealing with some measure of spermatogenic repair although histologic evidence of such repair was not evident. A clear cut evaulation of the testicular picture in terms of the content of nucleic acids is somewhat difficult in this series owing to the variable cellular content of the tubules. It can be stated, however, that the Sertoli cells were a constant feature of the tubules, that some of the tubules contained flattened basal cells and that both of these cells were significantly basophilic. Since the spermatoids and sperm contain little cytoplasm it would appear that the Sertoli cells and basal cells account for the majority of the PNA.

The increases in PNA concentration in the cryptorchid groups are not excessively large. Since the cell types which are present in the tubules contain considerable cytoplasm and since the Leydig cells show increased basophilia one might have expected higher values. Apparently the Sertoli cells in these testes are not sufficiently rich in PNA to provide the substantial increases seen in some of the other experimental series and the Leydig cells, since they are closely grouped and relatively more numerous may give the illusion of increased basophilia.

The concentrations of DNAP in the testes from hypophysectomized and estrogen-treated animals were double those found in normal adult animals. This is easily understood when we note that only cells whose nuclei possess diploid numbers of chromosomes were present.

Considerable variation was seen in the concentrations of DNAP in the testes of x-irradiated animals. However, if we take into account the variations which were encountered in the histologic condition of these testes the values for concentration of DNAP are seen to be consistent with the histologic picture. To assess this relationship more accurately it would be necessary to study a large series of animals in which individual analyses would be correlated with the varied histologic patterns in the same testes.

In the two groups of cryptorchid animals the values for the concentrations of DNAP present a somewhat baffling problem. All of the cells contain diploid numbers of chromosomes. Thus one would expect the concentrations of DNAP to be double the normal adult value, as was the case for hypophysectomized and estrogen-treated rats. Actually the concentrations were between the values observed for hypophysectomized and estrogen-treated groups and for normal adult animals. Since the Feulgen reaction in the testes of hypophysectomized and estrogen-treated animals is similar to that seen in normal adult testes it is possible that the Sertoli cells, which appear to be the predominant type of cell in the testes of cryptorchid rats, do not have as high a content of DNA as do the spermatogonia and spermatocytes which are abundant in the testes of hypophysectomized and estrogen-treated rats. That these latter cells contain larger amounts of DNA than are present in other intratubular cells has been shown by Caspersson's (1933, 1936) studies utilizing absorption microspectrophotometric techniques.

The concentration of ASP in the testes of hypophysectomized animals paralleled that of PNAP both being higher than the corresponding values for normal adult testes and, as noted earlier for PNAP, are probably related to the types of cells which are present. On the other hand an even higher value for ASP was seen in the estrogen-treated group. A suggestion of an effect of estrogen upon constituents of the acid soluble phosphorus fraction may be found in the observations of Dirscherl and Hauptmann (1950) who reported that estrogens stimulate anaerobic glycolysis and respiration in liver slices and of McShan, Meyer and Erway (1947) and Case and Dickens (1948) who found that some estrogens inhibit the succinoxidase and cytochrome oxidase systems.

In the x-irradiated animals the ASP concentration closely paralleled the values for PNAP and therefore the comments made for PNAP apply in this case.

The concentration of ASP in the testes of eighty-day cryptorchid rats was somewhat higher than in normal adults while in the two hundred-day cryptorchids the concentration was similar to the normal value. We are unable to offer an explanation for these variations.

The observation that the concentration of the LP fraction in the testes of hypophysectomized and estrogen-treated rats does not differ from the normal value would seem to confirm the reports of Sinclair (1940, 1941) that the testicular phospholipids are inert.

The testes of x-irradiated animals and of the two groups of cryptorchid animals showed concentrations of LP which were significantly higher than the corresponding value for normal adult testes. It is noteworthy that the Sertoli cells are a constant feature as well as the predominant type of cell in these groups and it might be offered as a possible explanation that the Sertoli cell contains a larger quantity of phospholipid. On the other hand the experimental conditions imposed in the cryptorchid and x-irradiated animals may possibly have altered directly the phospholipid content. It is obvious, however, that the present status of our knowledge regarding phospholipid metabolism renders extremely difficult the proposal of any sound interpretation of these observations.

The absolute values for all fractions in the experimental series were lower than the normal adult values. This is not surprising since the testes of all of the experimental animals were much smaller than normal and had various types of cells missing from their seminiferous tubules.

SUMMARY AND CONCLUSIONS

The testes of hypophysectomized, estrogen-treated, x-irradiated and cryptorchid rats were analyzed for pentose nucleic acid phosphorus, desoxypentose nucleic acid phosphorus, total nucleic acid phosphorus, acid soluble phosphorus and lipid phosphorus. Observations also were made on routine histologic preparations and on cytochemical preparations with the Feulgen reaction and for cytoplasmic basophilia. The latter two procedures are considered to be related to desoxypentose nucleic acid and pentose nucleic acid, respectively.

In testes from hypophysectomized and estrogen-treated animals the concentrations of all phosphorus fractions, except the lipid fraction, showed definite increases over the normal adult values.

In testes from x-irradiated animals moderate increases in the concentrations of all phosphorus fractions were seen.

In the cryptorchid rats a striking increase in the concentrations of the lipid fraction and a definite increase in the concentration of desoxypentose nucleic acid phosphorus were observed. The other fractions in testes from these animals showed only moderate increases in concentration.

The absolute values for all fractions in each of the experimental series were considerably lower than the equivalent values for normal adult animals. This is in keeping with the reduced size of the testes.

The concentrations of the various phosphorus fractions in the testes of hypophysectomized and estrogen-treated animals showed rather definite patterns which appeared to depend upon the types of cells which are present in such testes. On the other hand the values for the concentration of the several phosphorus fractions in testes of x-irradiated and cryptorchid animals present certain inconsistencies which may be due to physical and/or chemical alterations which are superimposed on such changes as may have occurred as a result of alterations in the cell populations. Further evaluation of these factors and their influences will require additional and more detailed studies.

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NOTES AND COMMENTS

SERUM ANTIDIURETIC SUBSTANCE (ADS) IN THE SHEEP AND COW

Although serum ADS has been found in rats (Birnie *et al.*, 1949); in humans (Lloyd and Lobotsky, 1950; Hawker, 1952a and 1952b); and in the kangaroo rat (Ames and van Dyke, 1952) a quantitative estimation of it in the sera of sheep and cows has not previously been reported.

This note reports the finding of serum ADS in sheep and cattle.

PROCEDURE

The method of Birnie *et al.* (1949)for measuring ADS in rats' sera was used in these experiments; the ADS units have been quantitated in terms of milliunits of pitressin *(Parke Davis pitressin 20 u. per ml.). Serum ADS was assayed in the following groups:*

A. Sheep
  (1) Non-pregnant, non-lactating ewes (8 animals)
  (2) Pregnant, non-lactating ewes (4 animals)
B. Cattle (Dairy and Beef)
  (1) Non-pregnant, non-lactating dairy cows (8 animals)
  (2) Non-pregnant, lactating dairy cows (8 animals)
  (3) Pregnant, non-lactating dairy cows (8 animals)
  (4) Pregnant, lactating dairy cows (6 animals)
  (5) Dairy bull (1 animal)
  (6) Non-pregnant, non-lactating beef cows (3 animals)
  (7) Beef bull (1 animal)

The dairy cows were graded into Grades I, II and III, according to their capacity to produce milk. The Grade I cows belonged to a record-holding herd for milk production in Queensland, Australia.

TABLE 1. ADS CONCENTRATION IN SHEEP DURING NON-PREGNANCY AND PREGNANCY

Species	Type	No. assayed	No. of assays	mμ ADH/ml. serum	mμ ADH/ml. (mean)
Sheep	non-pregnant & non-lactating	8	8	0.00 0.50 0.35 0.75 0.30 2.00 2.10 1.25	1.16
Sheep	pregnant & non-lactating	4	4	4.60 5.85 6.10 3.85	5.10

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RESULTS

A. Serum ADS in Sheep

Serum ADS was found in both the non-pregnant and pregnant sheep, with a higher concentration occurring during pregnancy, as shown in Table 1.

B. Serum ADS in Cattle

An antidiuretic substance was also determined in the sera of the dairy and beef cattle examined, and the results are presented in Table 2.

TABLE 2. ADS CONCENTRATION IN DAIRY AND BEEF CATTLE DURING NON-PREGNANCY, PREGNANCY AND LACTATION

Cattle	Type	No. assayed	Grade	mμ ADH/ml. serum	Mean	Reproductive state
Dairy	non-pregnant & non-lactating	2	1	4.00 3.20	3.60	nulliparous pre-puberal
	non-pregnant & non-lactating	3	1	5.40 5.65 5.05	5.37	nilliparous post-puberal
	non-pregnant & lactating	4	1	7.70 5.80 7.10 8.00	7.15	parous
	pregnant & non-lactating	4	1	5.90 7.15 7.50 7.55	7.02	—
	non-pregnant & non-lactating	4	2	6.00 5.05 7.35 7.70	6.52	parous
	non-pregnant & lactating	4	3	4.95 3.85 5.80 3.30	4.47	parous
	pregnant & non-lactating	4	3	6.20 4.15 5.00 5.15	5.12	—
	pregnant & lactating	6	1	7.25 4.35 3.20 5.95 5.75 5.30	5.30	—
	Bull	1	1		3.45	—
Beef	non-pregnant & non-lactating	2		4.30 3.40	3.85	nulliparous post-puberal
	Bull	1			2.90	—

Reference to Table 2 shows that in any type examined the better the dairy cow is graded for milk production, the higher is her serum ADS level.

SUMMARY AND CONCLUSIONS

(1) A substance possessing antidiuretic properties (ADS) is present in the freshly drawn sera of sheep and cattle.

(2) In ewes the serum ADS level is higher during pregnancy than in the non-pregnant state.

(3) In dairy cows of Grade I milking strain the serum ADS level is lowest in prepuberal heifers, and shows increments with sexual maturity, pregnancy and lactation.

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SERUM ANTIDIURETIC SUBSTANCE (ADS) IN THE HUMAN FEMALE

A substance possessing antidiuretic properties (and henceforth referred to here as ADS) was found in the serum of rats by Birnie *et al.* (1949); in the human by Lloyd and Lobotsky (1950), and by Hawker (1952a and 1952b); and in the kangaroo rat, Dipodomys merriami, by Ames and van Dyke (1952). Birnie *et al.* (1949) consider that ADS found in rat serum is probably identical with the antidiuretic hormone (ADH) produced by the posterior pituitary gland.

This paper reports the finding of ADS in the adult human female during the normal menstrual cycle, pregnancy and the immediate post partum period.

PROCEDURE

Serum ADS was assayed according to the method of Birnie *et al.*, (1949). A graph was constructed correlating ADS units with milliunit concentrations of freshly prepared

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pitressin (Parke Davis. 20 U. per ml.). Serum ADS was assayed in the following groups:

(1) Healthy non-pregnant females—mostly (medical) students—aged between 17–22 years; in all, 65 assays were done on 28 individuals at different times during the menstrual cycle.

(2) Healthy pregnant women—usually from the 10th or 12th week following the last menstrual period; a total of 61 assays were performed on 24 women.

(3) Healthy women in immediate post partum period; 50 assays were done on 11 women in the first 4–5 days after delivery.

RESULTS

This work shows that ADS is present in the serum of the healthy, cyclically menstruating female. Figure 1 shows clearly that during the menstrual cycle the serum ADS level is most commonly 0–1 mμ ADH/ml. of serum; the arithmetic mean of the 65 assays examined was 1.43 (S.D. ± 1.18) mμ ADH/ml. of serum. Figure 1 also reveals that during pregnancy the serum ADS level is higher than during the menstrual cycle, and the distribution is wider. Here the serum ADS level is most commonly 5–6 mμ ADH/ml. of serum; the mean of the 61 assays was 4.53 mμ (S.D. ± 1.34 ADH/ml. of serum.

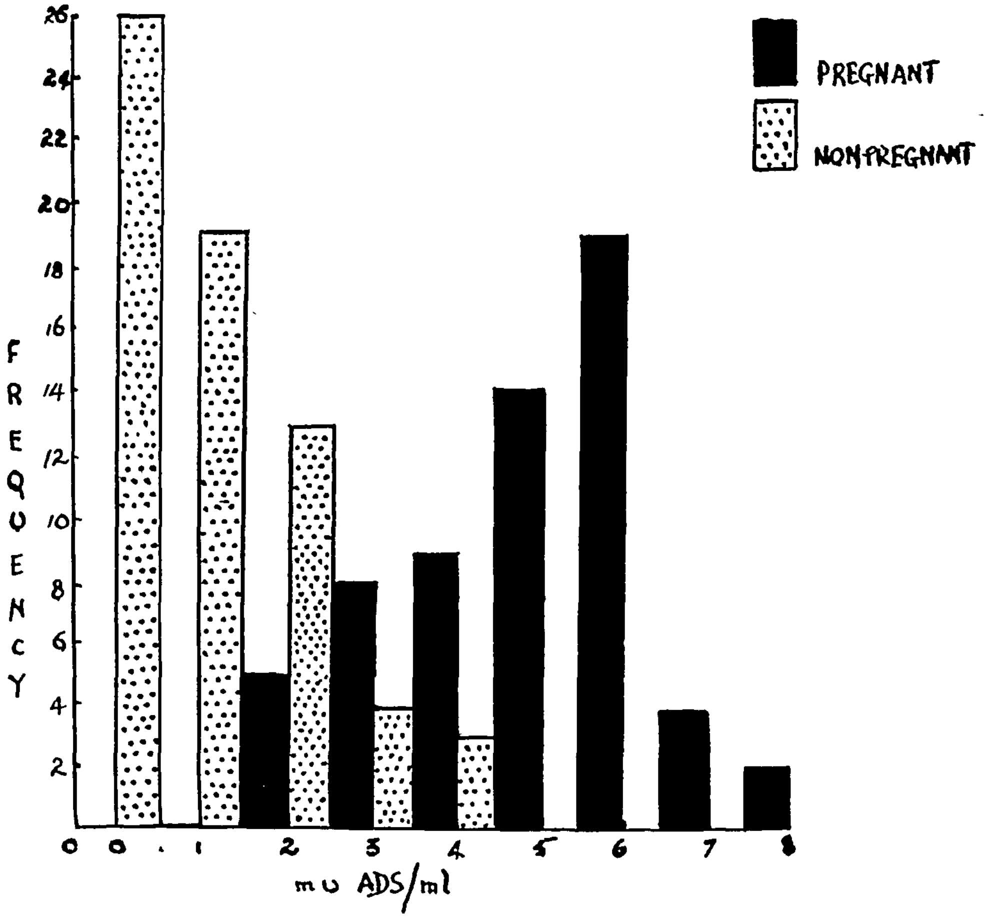

FIG. I. Histogram of serum ADS levels in pregnant and non-pregnant women.

The serum ADS levels in the weeks of the menstrual cycle are expressed in Table 1.

TABLE 1. ADS CONCENTRATION DURING THE MENSTRUAL CYCLE

		Milliunits of ADH/ml. serum	
Menstrual Cycle	Whole Cycle	1.43 (S.D. ±1.18)	Mean of 65 assays
	0– 7 days	1.48	Mean of 17 assays
	8–14 days	0.95	Mean of 10 assays
	15–21 days	1.72	Mean of 12 assays
	22–28 days	1.45	Mean of 15 assays

Some results are not included, as a record of the time in the menstrual cycle, when the assays were done, was not obtained.

The serum ADS levels during pregnancy are presented in Table 2, from which it will be seen that the level appears to remain comparatively steady throughout pregnancy with perhaps a slight over-all upward trend as pregnancy advances.

TABLE 2. ADS CONCENTRATION DURING NORMAL PREGNANCY AND FIRST DAY POST PARTUM

Time	Milliunits ADH/ml. serum (mean)	
1st Trimester	3.69	(4 assays)
2nd Trimester	4.21	(15 assays)
3rd Trimester	4.37	(21 assays)
Last month of pregnancy	4.37	(9 assays)
1st day post partum	5.50	(11 assays)

Some results are not included in the table because a record of the period of gestation was not obtained.

Immediately after parturition the serum ADS levels are higher than are found in non-pregnant women, and reference to Table 3 will show that a correlation appears to exist between the level of serum ADS and the onset of lactation. Here there occurred an initial post partum fall in the serum ADS level, and lactation began within a few hours after the serum ADS approached or reached its lowest recorded initial level. In no instance did lactation begin before the *initial* fall in the serum ADS level.

TABLE 3. ADS CONCENTRATION AND ONSET OF LACTATION

Case	Days after parturition				
	Milliunits ADH/ml. serum				
	1	2	3	4	5
1	7.0	5.85	5.0	*4.15*	5.2
2	4.9	4.45	*4.0*		
3	3.45	4.85	*2.35*	6.15	
4	*2.5*	3.15	3.35	5.1	
5	5.95	5.2	*2.9*	3.0	5.1
6	4.45	4.3	*3.35*	7.1	2.3
7	4.4	3.84	*0*	7.2	3.9
8	7.85	*3.3*	5.3		
9	5.5	*5.15*	7.2	2.95	4.1
10	5.6	5.6	5.85	*3.45*	3.9
11	5.9	*3.1*	4.9	2.75	5.45

Italicized figures = Lactation commenced within hours of this point.

The mean concentrations of ADH/ml. of serum for the eleven cases on the first day post partum and at the onset of lactation were 5.50 (S.D. ±1.29) and 3.11 (S.D. ±1.39) respectively. (First post partum reading for Case No. 4 excluded as lactation commenced on the first day post partum.)

SUMMARY AND CONCLUSIONS

(i) A substance possessing antidiuretic properties (ADS) is present in the freshly drawn serum of women during the normal menstrual cycle, normal pregnancy and early post partum period.

(ii) The serum ADS level is higher during pregnancy than during the menstrual cycle.

(iii) It is postulated that this high level during pregnancy accounts for some of the water retention of pregnancy.

(iv) A correlation appears to exist between the level of serum ADS after parturition and the onset of lactation. In each of the eleven cases examined, lactation always began after an initial fall in the serum ADS level.

Acknowledgements

I am grateful to the National Health and Medical Research Council (Australia) for their grant which enabled this work to be undertaken.

I wish to thank Professor W. V. Macfarlane of the Department of Physiology and Professor, H. J. G. Hines of the Department of Biochemistry, University of Queensland, *for their many helpful suggestions and criticisms during the course of this study.*

I am also indebted to Dr. R. Salter and Dr. K. G. Cockburn of the Brisbane Women's Hospital for their co-operation and I am pleased to acknowledge the technical assistance given to me during the course of these studies by Miss Joan Hunter, Department of Physiology, University of Queensland.

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THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.

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THE EFFECT OF ANDROGENIC HORMONES UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE INJECTIONS AND UPON THE ANTI-INFLAMMATORY ACTION OF CORTISONE[1]

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ZIZINE, Simpson and Evans (1950) reported that testosterone brought about a significant degree of adrenal repair in hypophysectomized animals. Since cortisone produces adrenal atrophy which resembles in many respects that seen after hypophysectomy, it was of interest to determine whether androgenic hormones would antagonize this effect of cortisone.

MATERIAL AND METHODS

Young adult male Holtzman rats were used in this study, except for the experiments where hypophysectomized rats were employed; the latter were purchased from the Hormone Assay Laboratories, Inc., Chicago. Steroids were injected in saline suspension subcutaneously, once daily. Some experiments were performed with testosterone propionate, but the androgen employed in most of the study was 17(α)-methyl-Δ^5-androstene-3(β)-17(β)-diol (methyl-androstenediol or mestenediol)* (M.A.D.). The cytochemical methods used in this investigation have been described elsewhere (Stebbins, 1951a).

RESULTS

Effect of testosterone in cortisone-treated rats. Table 1 gives the results of experiments in which cortisone was injected daily for a period of 9 days. Although adrenal atroply is not maximal within that period of time, it

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[1] Part of this work was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Omaha, Nebraska, October 15–17, 1951, and an abstract appears in the Proceedings of that meeting (*J. Pharmacol. Exper. Therap.* 103: 366. 1951).

* The authors gratefully acknowledge generous supplies of methylandrostenediol through the courtesy of Dr. K. W. Thompson, Organon, Inc., Orange, N. J.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE. TREATMENTS CONTINUED FOR 9 DAYS, MEAN VALUES FOR GROUPS OF 8 RATS

Treatment	Body weight		Thymus, mg.	Adrenals	
	Initial, g.	Final, g.		mg.	Mg./100 g. body wt.
Cortisone acetate 1.5 mg.	107	114	65	14.5	12.8
Cortisone acetate 3 mg.	98	104	24	14.1	13.5
Cortisone acetate 1.5 mg.+testosterone propionate 2.5 mg.	101	119	26	19.2	16.3
Cortisone acetate 3 mg.+testosterone propionate 2.5 mg.	109	108	18	19.0	17.8
Untreated controls	109	165	522	29.9	18.1

has been shown that most of the atrophy produced by cortisone occurs within the first 10 days (Winter, Silber and Stoerk, 1950). In the present experiment, there was not much difference in the degree of adrenal atrophy produced in 9 days by 1.5 mg. and by 3 mg. of cortisone acetate. Testosterone propionate, when given in conjunction with cortisone, prevented the adrenal atrophy to a considerable extent. It did not, however, prevent the suppression of body growth or atrophy of the thymus which is characteristic of cortisone-treated rats.

Effect of methylandrostenediol in cortisone-treated rats. When M.A.D. was given in graded doses in combination with 2.5 mgm. daily of cortisone acetate, a degree of prevention of adrenal atrophy was obtained which was directly related to the dose of M.A.D. employed up to 0.5 mgm. daily (Table 2). 0.05 mg. of M.A.D. produced no noticeable effect, but when 0.5 mg. was given to the cortisone-treated rats, the adrenals remained with-

TABLE 2. EFFECT OF GRADED DOSES OF METHYLANDROSTENEDIOL (M.A.D.) UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE. TREATMENTS CONTINUED FOR 19 DAYS (EXPERIMENT 1) OR 18 DAYS (EXPERIMENT 2); MEAN VALUES FOR GROUPS OF 9 TO 10 RATS

Treatment	Experiment No.	Body weight		Thymus, mg.	Adrenals	
		Initial, g.	Final, g.		Mg.	mg./100 g. body wt.
Cortisone acetate 2.5 mg.	1	106	92	23	13.0	14.3 ±0.66*
Cortisone acetate 2.5 mg.	2	120	111	23	12.9	11.8 ±0.59
Cortisone acetate 2.5 mg. +M.A.D. 0.05 mg.	1	113	106	22	14.7	13.9 ±0.60
Cortisone acetate 2.5 mg. +M.A.D. 0.1 mg.	1	106	99	20	15.0	15.1 ±0.68
Cortisone acetate 2.5 mg. +M.A.D. 0.25 mg.	1	111	98	20	15.8	16.2 ±0.89
Cortisone acetate 2.5 mg. +M.A.D. 0.5 mg.	2	115	116	25	23.7	20.5 ±1.45
Methylandrostenediol 0.5 mg.	2	125	188	381	43.8	24.2 ±1.83
Uninjected controls	1	111	190	435	39.3	21.3 ±1.81
Uninjected controls	2	125	198	440	42.7	21.8 ±1.07

* Standard error.

in the limits of normal size for rats of corresponding body weights. Higher doses of M.A.D. (not shown in the table) produced no greater effect upon adrenal weight. Thymus atrophy was virtually complete in all the animals treated with cortisone regardless of whether or not M.A.D. was also administered. The M.A.D. did not counteract the suppression of body growth by cortisone. M.A.D. by itself, in a dose of 0.5 mg. daily for 18 days produced about a 13 per cent decrease in thymus weight, but body weight and adrenal size were not significantly affected.

Histological examination of the adrenal glands of these animals demonstrated that the changes produced by cortisone could be largely prevented by concurrent administration of M.A.D. Cortisone produced a decrease in width of the cortex due to diminution in size of the cells of the zonae fascicularis and reticularis (Fig. 1). Cytoplasmic lipid (Fig. 7) and birefringent material (Fig. 11) were also largely lost. The glomerulosa remained largely unchanged except for a possible slight widening, and the presence of coarse birefringent particles. M.A.D. in doses of 0.5 mgm. daily prevented these changes (Figs. 2, 8, and 12), and the adrenals of the animals presented a microscopic picture similar to that of uninjected controls. No microscopic changes were observed in the adrenals of rats treated with M.A.D. alone.

TABLE 3. EFFECT OF CORTISONE AND METHYLANDROSTENEDIOL UPON ADRENAL WEIGHT IN HYPOPHYSECTOMIZED RATS

Treatment	Mean body weight, g.		Mean adrenal weight
	Initial	Final	Mg. ±S.E.
I. Cortisone 2 mg. daily	175	125	13.79 ± .50
II. M.A.D. 3 mg. daily	180	168	15.50 ± .83
III. Cortisone+M.A.D.	177	136	20.85 ± .94
IV. Uninjected controls	174	160	15.44 ± .75

Effect of cortisone and methylandrostenediol upon hypophysectomized rats. Rats that were hypophysectomized about 4 days were divided into groups as shown in Table 3. This experiment was performed twice, but as the results were identical in the two experiments, the data have been combined in the table. Each figure in Table 3 represents the mean of 16 to 20 rats. Treatment was continued for 18 days. The dose of M.A.D. was higher than that found in the preceding experiment to be necessary for optimal effects. Indeed, probably not all the administered androgen was absorbed, as deposits were found at injection sites when the rats were sacrificed. However, sufficient M.A.D. was absorbed to have a pronounced effect in combination with the cortisone.

None of the treatments succeeded in preventing the loss in body weight following hypophysectomy. Indeed, those treated with cortisone lost significantly ($P<0.01$) more than did the untreated controls and treat-

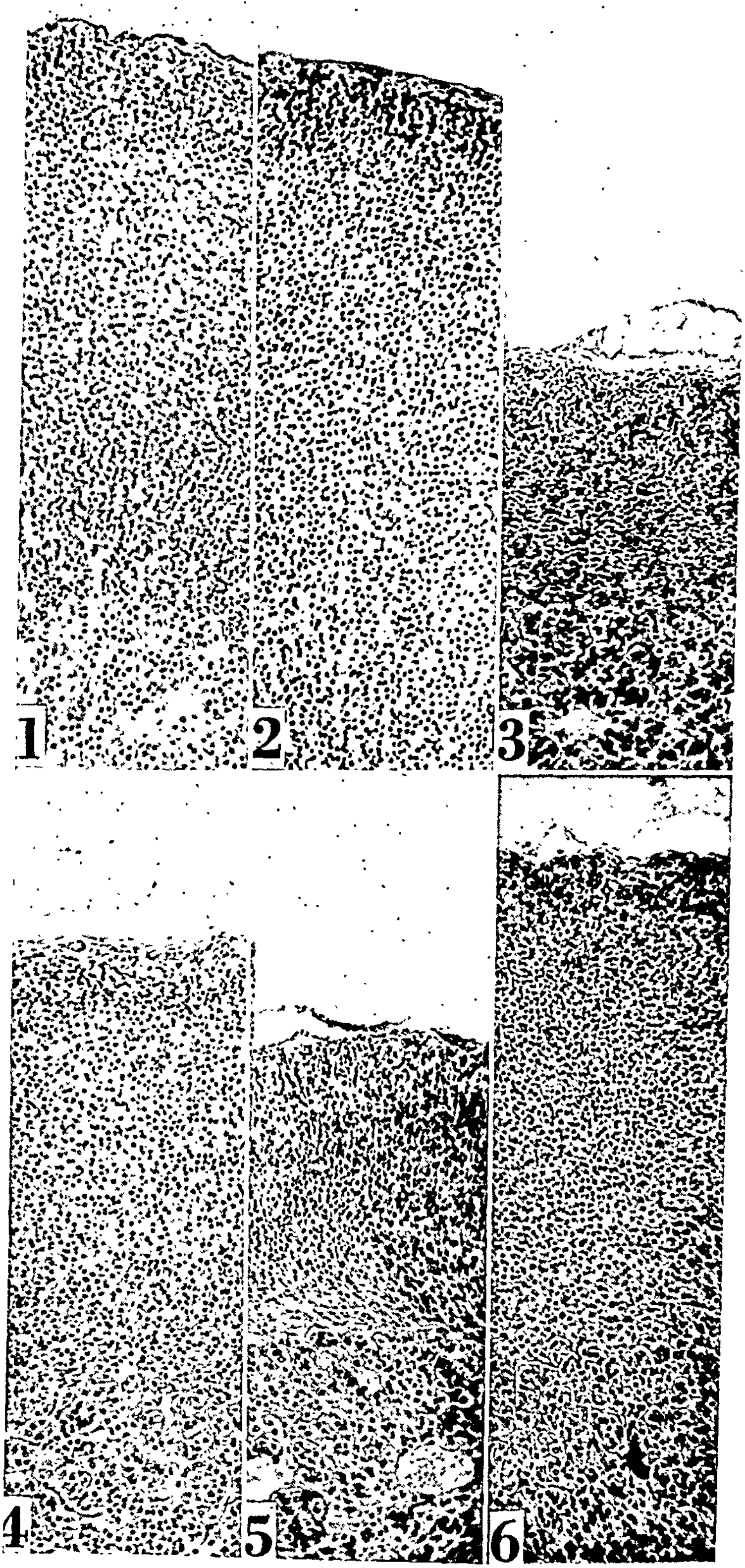

PLATE I

ment with the androgen did not prevent this excessive weight loss (P for difference in body weight loss, Groups I and III >0.05). M.A.D. alone did not significantly alter the weight loss in comparison with uninjected *controls* ($P>0.05$). *Therefore, only the cortisone affected weight loss in the* hypophysectomized rats; this loss in weight was greater than that commonly seen in intact rats treated with a similar dose of cortisone.

Effects on adrenal weights were very different from effects on body weight. Neither cortisone nor M.A.D. restored the adrenals of the hypophysectomized rats, but the combination of the two steroids restored the adrenals almost to the size of those observed in intact rats of comparable body weights.

The atrophy of the adrenals in the hypophysectomized rats receiving cortisone is shown in Figure 3, while Figure 4 demonstrates the repair occurring as a result of combination therapy of the two steroids. The atrophy in the presence of cortisone alone was not significantly greater than *that seen in uninjected hypophysectomized controls, in confirmation of* Stebbins (1951b). The extremely small size of the cells of the inner zones of the cortex of these atrophic glands (Fig. 3) was due to loss of cytoplasm, particularly of cytoplasmic lipid; the glomerulosa, in contrast, was thickened and showed a heavy deposition of lipid (Fig. 9). M.A.D. alone did not effectively restore the normal histological picture. The cells of the inner zones in these rats contained large vacuoles in which no lipid could be demonstrated. Considerable repair of the adrenal cortex could be seen in

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PLATE I. PARAFFIN SECTIONS OF RAT ADRENALS ×108. HEMATOXYLIN-EOSIN

FIG. 1. Adrenal of intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. The cells of the inner cortical zones are reduced in size due to loss of cytoplasmic lipid. The glomerulosa appears slightly wider than normal.

FIG. 2. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. methylandrostenediol for 18 days. Inner cortical cells contain more cytoplasmic lipid than in Fig. 1. The glomerular zone is essentially normal.

FIG. 3. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate for 18 days. Atrophic cells of inner cortex have lost lipid and nuclei are small and closely packed. Glomerulosa appears thickened.

FIG. 4. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Cells of zonae fascicularis and reticularis are larger and contain more cytoplasmic lipid and the glomerulosa is thinner than in Fig. 3.

FIG. 5. Intact rat injected daily with 2.5 mg. of cortisone acetate for 32 days. The section gives evidence of severe contraction of inner cortex with virtual extinction of cellular lipid and small closely arranged nuclei. Glomerulosa appears thickened.

FIG. 6. Intact rat injected with 2.5 mg. of cortisone acetate for 31 days and 0.5 mg. of methylandrostenediol daily from 18th to 31st day. Section displays a much wider inner cortex due to presence of lipid and pink-staining cytoplasm. Subglomerular and outer fascicular cells appear more closely packed than cells deeper in cortex.

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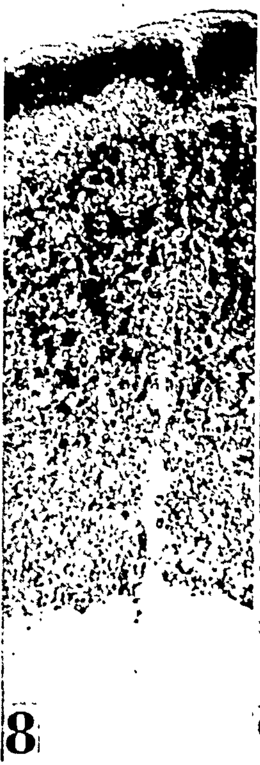
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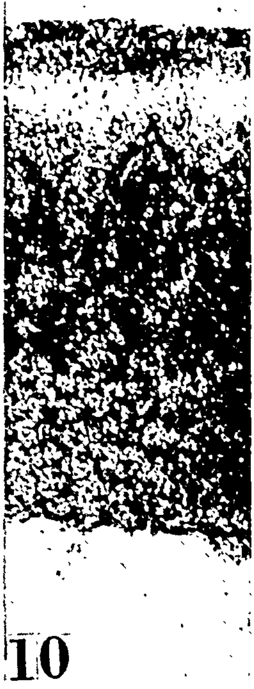
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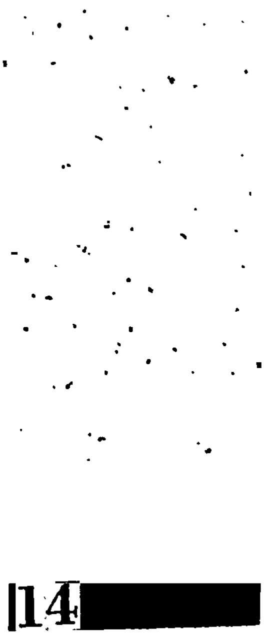
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PLATE II

the animals receiving combined therapy with the two steroids (Fig. 4). The adrenal cortices of these animals were much wider than those of untreated controls, or of those receiving either steroid alone. The cells were larger and more distinct and contained considerably more lipid (Fig. 10), while the birefringent particles were finer and more diffusely arrayed (cf. Figs. 13 and 14). In these respects the glands more nearly resembled those of intact rats. In general, the histological study confirmed the data obtained on adrenal weights.

Reversal of cortisone-induced adrenal atrophy. Having determined that M.A.D. could prevent cortisone-induced atrophy, and that the combination of steroids was effective in hypophysectomized rats, we next attempted to determine whether M.A.D. could restore the adrenals of intact rats in which atrophy had already been produced by cortisone, and also whether M.A.D. could hasten recovery of the adrenals after discontinuance of cortisone injections. Accordingly, 78 male rats weighing about 95 grams were injected with 2.5 mg. of cortisone daily for 17 days. These animals were then divided into groups of six rats, as shown in Table 4. In some groups (Ia to Id) cortisone was continued for varying lengths of time to obtain control figures on the degree of atrophy produced by this steroid.

Plate II. Distribution of Lipid and Birefringent Material in Frozen Sections of Rat Adrenals×108. Sudan Black B.

Fig. 7. Adrenal of intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. Shrunken cells of inner cortex have lost appreciable amount of lipid.

Fig. 8. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Larger cortical cells are laden with lipid.

Fig. 9. Hypophysectomized rat treated with 2.5 mg. of cortisone acetate for 18 days. Lipid is lost from outer area of inner cortex. Thickened glomerulosa contains large amount of cellular lipid.

Fig. 10. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Large cells of fasciculata and reticularis are laden with lipid. Thinner glomerulosa shows less lipid than in Figure 9.

Fig. 11. Intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. Coarse birefringent accumulations appear in glomerulosa with very fine particles in inner cortex. Subglomerular zone between them is empty.

Fig. 12. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. of of methylandrostenediol. Fine birefringent particles appear in the glomerulosa. A mixture of fine and coarse particles are seen in the fasciculata and reticularis.

Fig. 13. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate for 18 days. The glomerulosa displays closely arranged fine particles. The atrophic cells of inner cortex contain for the most part very fine delicate birefringent particles.

Fig. 14. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Very fine particles of glomerulosa are more freely distributed than in Figure 13. Larger cells of inner cortex contain larger and more distinct birefringent material.

TABLE 4. REVERSAL BY METHYLANDROSTENEDIOL (M.A.D.) OF ADRENAL ATROPHY PRODUCED BY CORTISONE ACETATE

Treatment	Mean adrenal weight, mg.
I. Cortisone acetate 2.5 mg. daily for:	
a. 17 da.	11.2
b. 21 da.	11.5
c. 24 da.	11.7
d. 31 da.	10.6
II. Cortisone acetate 2.5 mg. daily for:	
a. 21 da., M.A.D. 0.5 mg. last 4 da.	15.0
b. 24 da., M.A.D. 0.5 mg. last 7 da.	14.2
c. 31 da., M.A.D. 0.5 mg. last 14 da.	15.4
III. Cortisone acetate 2.5 mg. for 17 da.:	
a. sacrificed 4 da. after last injection	16.3
b. sacrificed 7 da. after last injection	23.7
c. sacrificed 14 da. after last injection	27.7
IV. Cortisone acetate 2.5 mg. for 17 da., then cortisone discontinued and M.A.D. 0.5 mg. started:	
a. sacrificed 4 da. after last cortisone	17.6
b. sacrificed 7 da. after last cortisone	21.6
c. sacrificed 14 da. after last cortisone	25.4

In comparable groups (IIa to IIc) M.A.D. was given in conjunction with cortisone during the last portion of the period of cortisone treatment. Additional groups were studied to determine the rate of adrenal recovery after discontinuance of cortisone without (IIIa to IIIc) and with (IVa to IVc) M.A.D.

The data demonstrate that after cortisone had been injected for 17 days, no further adrenal atrophy took place when injections were prolonged (Groups Ia to Id). When adrenal atrophy was allowed to occur, and then M.A.D. was given along with continuing cortisone injections (Groups IIa to IIc), a significant degree of recovery occurred ($P=0.02$). This recovery did not completely restore the adrenals to normal size, however. After cortisone was discontinued, recovery of the adrenals took place at the same rate whether or not M.A.D. was administered during the recovery period (Groups III and IV); in each case, the difference between adrenal size in the "recovery" animals with and without M.A. D. was not significant ($P>0.05$).

The histological picture of adrenal repair shown in Figure 6 is taken from an animal of Group IIc. This is in contrast to the markedly atrophic adrenals of the animals which received cortisone for the same length of time without M.A.D. (Fig. 5, animal of Group Id). Recovery of the adrenal cortex after discontinuance of cortisone has been described in a previous communication (Winter, Silber and Stoerk, 1950). Essentially, the same restoration of the histological picture was seen in the present series. Histologically, there was not much difference between the recovery with and without M.A.D., although there seemed to be a slight difference in favor of the M.A.D.-treated rats after 4 days of recovery

TABLE 5. EFFECT OF CORTISONE ACETATE AND METHYLANDROSTENEDIOL (M.A.D.) UPON THE EDEMA PRODUCED BY INJECTION OF IRRITANT (0.1 CC. OF 0.5 PER CENT $AgNO_3$ SOLUTION IN RAT'S FOOT)

Treatment	Amount of edema*	
	3 hours	24 hours
Untreated controls	51.8	42.2
M.A.D.—3 mg. daily for 3 da.	42.5	48.7
Cortisone—1.5 mg. daily for 3 da.	33.8	30.8
M.A.D.—3 mg. +cort. ac. 1.5 mg. daily for 3 da.	25.0	27.2

* "Amount of edema" = difference in cross-sectional area of foot before and after irritant (see text).

(Group IVa compared to IIIa) and a slight difference in favor of those without M.A.D. in the later recovery groups (IVb and c compared to IIb and c). These differences were too small to be certain of their significance.

Effect of methylandrostenediol upon the anti-inflammatory action of cortisone. Since M.A.D. clearly antagonized the production of adrenal atrophy by cortisone, it was of interest to determine whether this androgen would also antagonize the anti-inflammatory effects of cortisone. Sely (1949) has shown that cortisone inhibits the local effect of irritants injected under the plantar surface of the rats' foot. We have employed a similar technique, using as an irritant 0.1 cc. of 0.5 per cent $AgNO_3$ solution, and measuring the edema 3 hours and 24 hours after the irritant. The animals were pretreated for 3 days with steroids subcutaneously. The results are given in Table 5. The "amount of edema" was calculated as follows: measurements of two diameters of the rat's foot were made by calipers at about the midmetacarpal line. Assuming the cross-section of the foot to be an ellipse, the cross-sectional area in sq. mm. was calculated. The difference between this area before and after injection of irritant was termed "amount of edema."

TABLE 6. EFFECT OF CORTISONE AND METHYLANDROSTENEDIOL (M.A.D.) UPON GRANULOMA FORMATION INDUCED BY SUBCUTANEOUS COTTON PELLETS IN RATS

Treatment	Per cent increment in dry weight cotton pellet
A. Systemic steroids	
Untreated controls	146 ±6.6*
M.A.D. 3 mg. daily for 6 da.	123 ±9.8
Cortisone acetate 2 mg. daily for 6 da.	70 ±5.6
Cortisone acetate 2 mg. +M.A.D. 3 mg. daily for 6 da.	70 ±3.5
B. Local Steroids	
Cortisone acetate 0.25 mg.	123†
Controls	162
M.A.D. 0.75 mg.	143
Controls	143
Cortisone acetate 0.25 mg. +M.A.D. 0.75 mg.	98‡
Controls	139

* Standard error.
† Difference between controls and treated animals, $P = 0.05$.
‡ Difference between controls and treated animals, $P < 0.01$.

It is apparent that M.A.D. alone had no effect upon the production of edema, and that cortisone was not less effective in presence of the androgen.

In another experiment, cotton pellets were implanted subcutaneously and the amount of granuloma tissue formed at the site of the pellet was determined by removing the pellet together with the granuloma around it after 6 days, according to the technique described by Meier, Schuler and Desaulles (1950). This reaction is inhibited by cortisone, either administered systemically or by local application (incorporation of the steroid in the pellet). In the test of the local action of the steroid, a control pellet is placed on one side of the animal, and a pellet containing steroid on the opposite side. Thus, each animal serves as its own control. Table 6 gives the results by both methods of administration. It is evident that M.A.D. does not have any influence upon the amount of granuloma formed, nor does it antagonize the inhibition of the reaction by cortisone.

Both these experiments make it clear that although M.A.D. antagonizes the effect of cortisone in producing adrenal atrophy, it does not antagonize the anti-inflammatory action of cortisone.

DISCUSSION

Although cortisone has proven to be useful in a variety of conditions, it exerts certain hormonal effects which do not contribute to its therapeutic value, and which, indeed, it would be desirable to avoid, if possible. Among these is adrenal atrophy. This investigation was undertaken with the thought that if one or more of these unwanted effects could be counteracted without diminishing the desired actions of the hormone, the usefulness of cortisone might be enhanced. This hope seems to have been at least partially realized in these animal experiments, although the therapeutic applicability of these results has not as yet been demonstrated. The hyperadrenocortinism produced in patients by large doses of cortisone resembles in many respects the findings in Cushing's syndrome. Albright (1942–43) found androgens to be of value in correcting many of the manifestations of this disease. Pearson (1951) also reported that testosterone would counteract some of the effects of cortisone on normal tissue.

Both testosterone and methylandrostenediol exerted a sparing action upon the adrenal cortices of animals treated with cortisone. Both these compounds are androgens, and both also have anabolic action as shown by growth-promotion and nitrogen balance tests (Kochakian, 1949; Homburger *et al.*, 1950). Methylandrostenediol, however, has been reported to be relatively high in the anabolic action typical of androgens, but low in androgenic potency (Gordan *et al.*, 1951). It is possible that the sparing action of these steroids on the adrenal cortex of cortisone-treated rats is a consequence of their anabolic action. In the present experiments, no such action could be demonstrated upon the growth-suppressing action of cortisone. However, it is not necessary to assume that steroids have a qualitatively similar action upon metabolic phenomena in all the tissues of the body; indeed, there is good evidence that cortisone has opposite metabolic effects upon different tissues (Kochakian and Robertson, 1951). It is therefore not unreasonable that our experiments might show an anabolic action of M.A.D. on the adrenals even though they did not demonstrate such action on the body as a whole.

In the intact animals, it might be argued that M.A.D. could act as a stress to effect the release of ACTH, and adrenal atrophy could be prevented by release of this hormone from the hypophysis. Alternatively, *M.A.D. might block the inhibiting effect of cortisone upon the release of* ACTH. The results in hypophysectomized rats deny both these alternatives. The repair of adrenals after hypophysectomy when both cortisone and M.A.D. are used, but not when either is administered alone leads to the surprising conclusion that both cortisone and the androgen are necessary for this result. Also, in the experiments on adrenal repair after establishment of adrenal atrophy by cortisone, M.A.D. brings about some repair if it is administered while cortisone is being given. On the other hand, when M.A.D. is given alone; i.e., after discontinuance of cortisone, adrenal repair does not proceed more rapidly than if no steroid is being given. Therefore, in this experiment as in the experiment with hypophysectomized rats, it appears that it is the combination of the two steroids which is effective against adrenal atrophy, rather than either one alone. On the other hand, no combination of the two steroids was used which gave adrenals larger than those of nomal intact rats (several dosage ratios were tried which were not included in the tables) nor did M.A.D. alone in intact rats produce large adrenals.

SUMMARY

1. Testosterone propionate or methylandrostenediol administered in conjunction with cortisone acetate in rats prevented the adrenal atrophy which occurred when cortisone alone was given. Adrenal cortices of rats given *both the androgen and cortisone were essentially normal in size and* histological appearance.

2. Combination therapy with methylandrostenedial and cortisone produced considerable repair of the adrenals of hypophysectomized rats. Neither steroid alone produced repair of adrenals in such animals.

3. Adrenals made atrophic by cortisone injection could be partially repaired by administration of methylandrostenediol while cortisone treatment was continued. However, adrenal recovery after discontinuance of cortisone was not hastened by injection of the androgen.

4. Methylandrostenediol administered with cortisone did not inhibit the anti-inflammatory action of cortisone.

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GONADOTROPINS OF THE PITUITARY GLAND AND THE URINE OF THE ADULT HUMAN FEMALE

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IN A previous paper (Bahn *et al.*, 1952), a study of the gonadotropins of the pituitary gland and the urine of the adult human male was made. The purpose of this paper is to present similar data on the pituitary gland and the urine of the adult human female.

I. MATERIALS AND METHODS

A. Animals. Male and female Wistar rats (30 to 40 days old) were hypophysectomized; injected, some with pituitary homogenate and others with urine concentrate; and subsequently studied as described previously.

B. Pituitary tissue. The pituitary gland of 2 adult human females was obtained within three to four hours following death. Neither of the patients presented any obvious endocrinopathy. Menstruation had occurred at regular monthly intervals. The ages of the patients were 38 and 46 years. There was no prolonged terminal illness. The combined wet weight of the anterior lobes of the pituitary glands was 1,219 mg.

C. Urine. A number of complete twenty-four hour specimens of urine were obtained from 5 healthy menstruating adult females during all phases of the menstrual cycle.

II. RESULTS

A. Pituitary homogenate in female animals. The results observed in hypophysectomized female rats receiving anterior pituitary homogenate are listed in Table 1. The data indicate that both follicle-stimulating (FSH) and luteinizing (LH, ICSH) hormones are present in the normal adult human female hypophysis. Neither early follicular growth nor repair of the ovarian interstitial "deficiency cells" was observed until a total dose of 3 mg. of the pituitary homogenate was given.

Uterine and ovarian enlargement occurred at a dose of 10 mg. The increase in ovarian weight was associated with the presence of many large cystic follicles. There was only slight luteinization of the granulosa cells while the interstitial and thecal cells had undergone marked lutein cell transformation. The response to 30 mg. of anterior pituitary homogenate

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TABLE 1. EFFECT OF HUMAN PITUITARY HOMOGENATE ON THE HYPOPHYSECTOMIZED FEMALE RAT

Animals			Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Dose (mg.)	mg.	mg./gm.	mg.	mg./gm.	F.D.	Corpora lutea	I.T.	
Hyp.	4	0	5.6	0.10	15.0	0.28	0	0	D	0
Intact	6	0	18.6	0.18	118.3	1.17	+++	+	R	+
Hyp.	4	1	8.0	0.12	21.8	0.32	0	0	D	0
Hyp.	3	3	9.2	0.14	22.9	0.35	+	0	R	0
Hyp.	4	10	38.8	0.54	98.8	1.37	++++	0	L	0
Hyp.	1	30	81.2	1.35	78.4	1.31	++++	+	L	+

Abbreviations: Hyp. = Hypophysectomized; mg./gm. = Organ weight per gram body weight; D = Deficient; R = Repaired; I.T. = Interstitial tissue; 0 = absent; + = Present; L = Luteinized; V.O. = Vaginal opening; F.D. = Follicular development.

was characterized by additional ovarian enlargement and the formation of numerous corpora lutea.

In terms of the previously defined units, there is approximately 1 unit of FSH and 1 unit of LH per 3 mg. of pituitary homogenate, or a total of about 200 units of both hormones per pituitary of the adult human female.

B. Pituitary homogenate in male animals. Table 2 lists the results on hypophysectomized male rats. At the 3 mg. dose level FSH activity was indicated by a doubling of testicular weight, an increase in tubular diameter and active spermatocytic proliferation as compared with the controls. Thirty milligrams of pituitary homogenate tripled the testicular weight, but did not completely restore the normal testicular histologic pattern. Only the testis of the intact control animals exhibited spermatozoa.

TABLE 2. EFFECT OF HUMAN PITUITARY HOMOGENATE ON THE HYPOPHYSECTOMIZED MALE RAT

Animals			Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Dose (mg.)	mg.	mg./gm.	mg.	mg./gm.	mg.	mg./gm.	M.T.D.	S.A.	St.	Sz.	I.T.
Hyp.	2	0	13.9	0.22	15.0	0.23	47.3	0.74	0.10	+	0	0	U.
Intact	5	0	62.2	0.58	41.0	0.38	132.2	1.22	0.22	++++	+	+	Di.
Hyp.	3	1	27.3	0.39	22.5	0.32	75.6	1.09	0.14	++	0	0	U.
Hyp.	3	3	45.8	0.64	41.1	0.57	115.4	1.61	0.16	+++	0	0	Di.
Hyp.	2	10	54.2	0.77	46.5	0.66	140.1	2.00	0.15	+++	0	0	Di.
Hyp.	3	30	52.7	0.68	60.1	0.78	163.9	2.13	0.16	++++	+	0	Di.

Abbreviations: M.T.D. = Mean tubular diameter (millimeters); S.A. = Spermatocytic proliferation; St. = Spermatid; Sz. = Spermatozoa; I.T. = Interstitial tissue; U. = Undifferentiated; Di. = Differentiated; Others = See table 1.

At the 3 mg. dose level LH activity was indicated by the appearance of well-differentiated Leydig cells. Nevertheless, indirect evidence of Leydig cell function was present at the 1 mg. dose level as judged by the weights and results of histologic examination of the prostates and seminal vesicles. The amount of homogenate necessary to repair the deficient Leydig cells as judged by histologic evidence was 3 mg. The same amount also brought about a distinct response in the tubular apparatus of the testis as judged by testicular weight, tubular diameter and spermatogenic activity. Again, roughly equal FSH and LH activities, about 200 units of each, are present in the adult female pituitary.

C. Urine in female animals. Table 3 shows the effect of a concentrate equivalent to 1,200 cc. of pooled urine from adult females. The presence of FSH was shown by the increased ovarian weight and the marked response of the ovarian follicles. The latter were large cystic structures without signs of luteinization. Ova were present within these follicles. Both ovarian and uterine enlargements were about equal to that brought about by 3 to 10 mg. of pituitary homogenate.

TABLE 3. EFFECT OF URINARY CONCENTRATE ON THE HYPOPHYSECTOMIZED FEMALE RAT

Animals				Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Cortisone	Concentrate	mg.	mg./gm.	mg.	mg./gm.	F.D.	Corpora lutea	I.T.	
Hyp.	2	0	0	7.3	0.11	16.3	0.23	0	0	D	0
Hyp.	3	+	0	8.7	0.12	16.3	0.23	0	0	D	0
Hyp.	5	+	+	20.2	0.30	76.8	1.15	++++	0	D	+
Intact	5	0	0	18.4	0.16	83.9	0.73	+++	+	R	+

Abbreviations: Same as in table 1.

The presence of LH was shown by the copious secretion of estrogen, resulting in uterine enlargement. However, since there was no repair of the interstitial tissue of the ovary, less than 1 unit of LH as previously defined was present in 1,200 cc. of urine.

D. Urine in male animals. Table 4 lists the weights of organs of hypophysectomized male rats receiving urinary concentrate. Little change occurred in the number or the size of the interstitial cells as compared to the hypophysectomized control animals. Nevertheless, the weight of the ventral prostate, epididymis and seminal vesicles was distinctly increased over that of the hypophysectomized controls. Moreover, microscopic examination of the ventral prostate and seminal vesicles gave evidence of

TABLE 4. EFFECT OF URINARY CONCENTRATE ON THE HYPOPHYSECTOMIZED MALE RAT

Animals				Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Cortisone	Concentrate	mg.	mg./gm.	mg.	mg./gm.	mg.	mg./gm.	M.T.D.	S.A.	St.	Sz.	I.T.
Hyp.	4	0	0	6.5	0.09	8.6	0.12	49.5	0.69	0.13	+	0	0	U.
Hyp.	5	+	0	6.6	0.10	8.9	0.14	41.9	0.66	0.13	+	0	0	U.
Hyp.	5	+	+	9.4	0.14	10.9	0.16	76.0	1.09	0.16	+++	+	0	U.
Intact	5	0	0	65.7	0.55	44.3	0.37	171.3	1.44	0.23	++++	+	+	Di.

Abbreviations: Same as in Table 2.

IN VITRO BIOASSAY OF CORTICOTROPHIN

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WE HAVE observed that pituitary adrenocorticotrophic hormone (corticotrophin), added *in vitro*, stimulates the formation of corticoids by isolated rat adrenals and that the stimulation is approximately proportional to the amount of corticotrophin added (Saffran, Grad and Bayliss, 1952). This observation has been adapted to the bioassay of corticotrophin.

METHOD

Four adult Sprague-Dawley rats, of either sex, are used for each assay. The adrenals are prepared as previously described (Saffran, Grad and Bayliss, 1952). Care must be taken to keep the pair of adrenals from each rat together during the preparation. Four adrenals, one from each pair, are weighed together, bisected, and placed into a Warburg vessel containing 2 ml. of the Krebs-Ringer-phosphate-glucose medium. The remaining four adrenals are then weighed, bisected, and placed into a second Warburg vessel. The flasks are attached to Warburg manometers, flushed with oxygen, and are shaken at 38° for 1 hour. At the end of this period (the preincubation period) the media from both flasks are removed with suction and discarded. Both flasks are then recharged with fresh medium, and a small volume (about 0.1 ml.) of the standard corticotrophin solution is added to one flask and the unknown corticotrophin solution to the other. Approximately the same activity of corticotrophin should be added to both flasks. The flasks are again flushed with oxygen and shaken at 38°, this time for 2 hours. At the end of this period the media are analyzed separately for corticoids by a slight modification of the method previously described (Saffran, Grad and Bayliss, 1952). The modification consists of passing the chloroform extract through filter paper instead of drying with sodium sulfate. The filtered chloroform extract is then taken to dryness as previously described.

The *amount* of corticotrophin is expressed as units of the standard preparation and mg. of the unknown per 100 mg. of adrenal tissue in the flask. The *activity* of the corticotrophin is expressed as μg. of corticoids produced by 100 mg. of adrenals in 2 hours.

The potency of the unknown sample is calculated as follows:

$$\text{Potency (units per mg.)} = \frac{\text{units of standard corticotrophin x activity of unknown}}{\text{mg. of unknown corticotrophin x activity of standard}}$$

The procedure is outlined in the sample protocol. In actual practice several levels of standard and unknown preparations are matched for each determination of potency.

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SAMPLE PROTOCOL

Comparing Armour's corticotrophin with the U.S.P. reference standard

	Standard	Unknown
Weight of 4 adrenals, mg.	48	45
Tissue added to flask	9:58 A.M.	10:00 A.M.
Preincubation period	10:10 to 11:10 A.M.	
Corticotrophin added	U.S.P.	Armour
ml.	0.1	0.1
units or mg./flask	0.025 unit	0.012 mg.
units or mg./100 mg.	0.053 unit	0.027 mg.
Second incubation period	11:30 A.M. to 1:30 P.M.	
Observed optical densities:		
225 mμ	0.285	0.285
240 mμ	0.260	0.254
255 mμ	0.174	0.167
Calculated O.D.	$.260-\frac{.285+.174}{2}=0.031$	$.254-\frac{.285+.167}{2}=0.028$
Total μg. corticoids	5.6	5.1
μg. corticoids/100 mg.	11.75	11.3

$$\text{Potency of corticotrophin, Armour}=\frac{0.053\times 11.3}{0.027\times 11.75}=1.93\text{ units/mg.}$$

EXPERIMENTAL

Extraction of corticoids

A methanolic solution of the residue from a chloroform extract of the medium alone absorbs light in the ultraviolet at the wave lengths used to measure the corticoids (Fig. 1). Although the absolute value of the blank absorption varies somewhat, the shape of the curve is almost invariably the same. Because the blank curve approaches a straight line it is possible to use the calculation of Allen (1950) to estimate the absorption at 240 mμ caused by the α,β-unsaturated ketonic grouping of the corticoids. The absorption curve of a chloroform extract of the incubation medium of rat adrenals kept at 2° for 2 hours in the presence of corticotrophin is very similar to the blank. Figure 1 includes the curve of an extract of the incubation medium of adrenals kept at 38° with corticotrophin present and the absorption curve of cortisone for comparison with the blank curves.

Attempts to eliminate the high blank absorption by replacing the chloroform with methylene chloride, ethyl acetate, heptane, or ether failed because these solvents either yielded higher blank values than chloroform or because they did not extract the corticoids from an aqueous medium.

Preincubation

In this paper "preincubation" means the incubation of the adrenals for

Rate of corticoid formation

The rate of formation of corticoids, in the presence or absence of added corticotrophin, is approximately constant for 4 or 5 hours (Fig. 3).

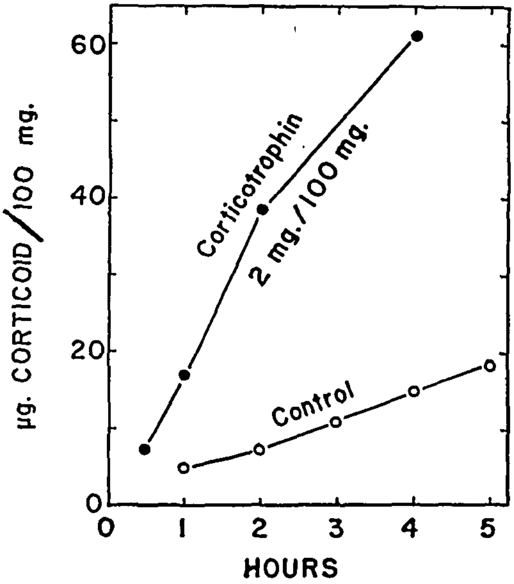

Fig. 3. Rate of formation of corticoids by bisected rat adrenals in the presence and absence of added corticotrophin.

Persistence of corticotrophin effect

Rat adrenals, incubated for 1 hour with corticotrophin, produce increased amounts of corticoids even after two thorough rinsings with the medium (Fig. 4).

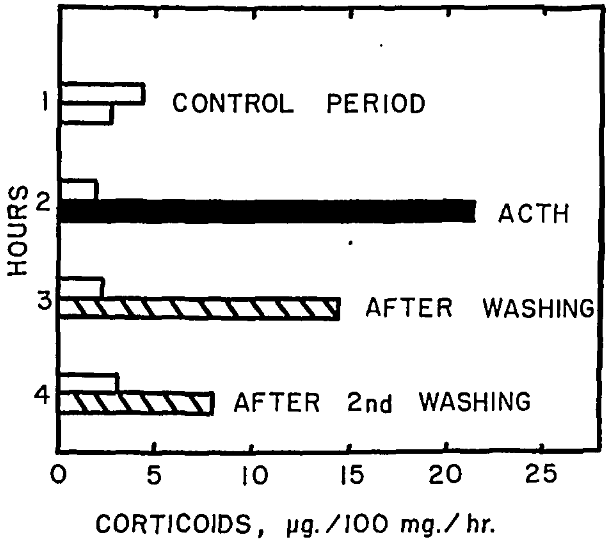

Fig. 4. Persistence of the effect of added corticotrophin after washing of the adrenal tissue.

Incubation in blood

Because of the reported greater production of corticoids by beef adrenals when perfused with whole blood than with plasma (Hechter *et. al.*, 1951), bisected rat adrenals were incubated in citrated homologous blood instead of the usual medium. In this experiment a mixture of 5% carbon dioxide in oxygen was used as the gas phase. At the end of 2 hours little or no corticoids were found in a chloroform extract of the blood (Table 3).

TABLE 3. EFFECT OF CHANGES IN CONDITIONS OF INCUBATION ON THE FORMATION OF CORTICOIDS

Conditions	Corticotrophin added	Corticoids formed	Oxygen consumption
	units/100 mg.	μg./100 mg. per 2 hrs.	μM/100 mg. per 2 hrs.
Citrated blood and 5% CO_2	0	4.1	
	1.7	3.4	
	0	0	
	1.7	9.4	
	0	0	
	1.6	0	
Oxygen	1.7	32.1	12.5
Air	1.7	23.9	3.9
Nitrogen	1.6	0	0
	1.7	0	0
Bisected	1.5	24.8	11.6
	1.7	30.0	12.2
Minced	1.5	28.9	7.6
	1.6	21.6	7.2
Homogenized	0	0	1.0
	1.0	0	1.2

N.B. These samples were *not* preincubated.

Oxygen requirement

The oxygen requirement of the system that forms corticoids was investigated by incubating rat adrenals in air and in nitrogen. The formation of corticoids was somewhat lower in air than in oxygen, but no corticoids were formed during the anaerobic incubation in nitrogen (Table 3).

Mincing and homogenization

Mincing the adrenal tissue with scissors does not affect the formation of corticoids, but there is no formation in an unfortified homogenate of adrenals (Table 3).

Agreement between duplicates

Four adrenals, one from each of four rats, form about the same amount of corticoids as the four remaining adrenals from these animals, when the two lots are incubated under the same conditions (Table 4).

TABLE 4. AGREEMENT BETWEEN DUPLICATE INCUBATIONS

	Corticotrophin added	Corticoids formed	
	units/100 mg.	µg./100 mg./2 hrs.	
Not preincubated	0	4.2	2.6
	0	1.6	1.9
	0	3.3	4.2
	1.0	12.6	9.9
	2.5	18.5	16.0
	2.6	20.3	16.1
Preincubated	0	5.8	4.6
	0	3.8	7.4
	0	3.0	1.5
	0.8	32.3	35.8

RESULTS

The *in vitro* bioassay method was used to determine the potency of several preparations of corticotrophin. The values agree very well with the estimated potencies based on the adrenal ascorbic acid depletion method of Sayers, Sayers and Woodbury (1948) (Table 5).

TABLE 5. POTENCY OF CORTICOTROPHIN PREPARATIONS BY THE *in vitro* BIOASSAY

Preparation	Estimated potency by *in vitro* assay	Claimed potency*
	U.S.P. units/mg.	U.S.P. units/mg.
Armour, lot J16609	1.6	2
	1.9	
	1.9	
Astwood, crude	1.6	2
	2.0	
	1.8	
Astwood, purified	50.1	60–100
	52.6	(average, 80)
	56.2	
	66.6	
Canada Packers, lot 2-C	3.1	3–4
	2.7	
	3.5	
Canada Packers, lot FU	3.0	3–4
Canada Packers, lot I	2.9	4–5
(deteriorating sample because of high moisture content)	2.7	

* Estimated by the method of Sayers, Sayers and Woodbury (1948). Values supplied by the donors of the corticotrophin preparations.

DISCUSSION

Although the optimum conditions for the *in vitro* assay method have not been established yet, it yields results that agree very well with the adrenal ascorbic acid depletion method. The *in vitro* method has several advantages. Firstly, the same animals are used to provide tissue for both the standard and unknown preparations of corticotrophin. Secondly, there is no need to maintain a supply of hypophysectomized animals because in the *in vitro* assay the adrenals are effectively removed from the influence of the pituitary. Thirdly, a known function of corticotrophin, namely, the control of the secretion of adreno-cortical hormones, is used as the basis of the assay. A disadvantage of the method is its relative insensitivity, but this is of little consequence when the potency of a preparation of corticotrophin is being determined.

The method is based on an assumption for which there is no direct evidence. It is, that a given group of adrenals, incubated together, will produce an amount of corticoids that is proportional to the amount of corticotrophin added. In the case of the adrenals (Fig. 2) that were not preincubated, the response to increasing amounts of corticotrophin seems to be more or less linear. After preincubation, the adrenal response curve is very steep, but for the assay it is assumed to be linear as well. That this assumption is valid to some extent is shown by the good agreement among replicate assays of the various preparations of corticotrophin (Table 5).

Both adrenals of a rat respond similarly to corticotrophin (Table 4). This allows the comparison of standard and unknown preparations of corticotrophin on similarly-reacting tissue. If homogeneous preparations of adrenal tissue can be shown to respond to corticotrophin like bisected adrenals, it would be possible to divide the tissue into more than two aliquots and allow the comparison of more than two samples using the same tissue. That this might be so is indicated by the response of an adrenal mince to corticotrophin (Table 3).

Preincubation increases the sensitivity of the adrenals to corticotrophin about 20–25 times. During the preincubation period a large proportion of the ascorbic acid is leached from the adrenal tissue (Saffran, Bayliss and Webb, 1951) and the tissue may have had the time to recover from any previous stimulation by corticotrophin in the animal. However, there is, as yet, no evidence for any explanation of the effect of preincubation.

The effect of corticotrophin on the adrenal *in vitro* persists after washing the tissue (Fig. 4). This observation is similar to that of Stadie and his group (Stadie, Haugaard and Marsh, 1951) on the *in vitro* effect of insulin. It is not possible now to state whether the corticotrophin is "bound" by the adrenal tissue.

SUMMARY

The stimulation by corticotrophin of the production of corticoids by bisected rat adrenals *in vitro* has been adapted to the assay of corticotrophin. The sensitivity of the method is greatly increased by the preincubation of the adrenal tissue without added corticotrophin for 1 hour, prior to exposure to corticotrophin. The range of the assay is approximately 0.04 to 0.1 U.S.P. units of corticotrophin per 100 mg. of adrenal tissue.

There is very good agreement between the *in vitro* bioassay and the adrenal ascorbic acid depletion method.

The stimulation by corticotrophin of the production of corticoids by rat adrenals persists after washing the tissue to remove the corticotrophin.

Corticotrophin stimulates the production of corticoids in an atmosphere of air as well as in oxygen, but the rat adrenal does not form corticoids in nitrogen.

An adrenal mince responds to added corticotrophin like bisected adrenals, but unfortified homogenates do not produce corticoids.

Acknowledgments

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NEUROHUMORAL CONTROL OF THE PITUITARY IN THE FOWL

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IN THE domestic fowl approximately 23 to 28 hours elapse between ovulation and the laying of the finished egg. During the intervening period the ovum acquires albumen in the magnum and shell membranes in the isthmal portion of the oviduct. This is accomplished in 4.5–5.0 hours and the remaining time is spent in the shell gland where the calcifierous shell is deposited. Warren and Scott (1935) showed that ovulation does not usually occur while there is an egg in any portion of the oviduct. These facts and the finding that, in hens laying daily, ovulation takes place within 30–60 minutes after oviposition, led to the assumption that an intricate timing mechanism may be involved to synchronize reproductive events in chickens. An observation in this laboratory that injury to the oviduct led to a profound upset in the laying cycle of hens caused more intensive analysis of the relationship between events in the oviduct and the endocrine function of the pituitary.

Rothchild and Fraps (1949) have shown that the ovulating hormone (LH) is released about 4 to 8 hours prior to ovulation. Thus, factors which may be involved either in stimulating or in inhibiting release of LH must exert their effects several hours prior to ovulation. Since there is only a relatively short interval between oviposition and the succeeding ovulation it becomes apparent that the events leading to ovulation usually occur while the egg is in the shell gland.

These observations led to the experiments reported here.[1] Initially, such irritants as bits of key chain, cork or pellets of paraffin were placed in the lumen of the magnum. These foreign bodies were attached to coarse surgical thread which in turn was passed through the wall of the oviduct and tied securely so as to prevent evacuation of the irritants from their original location in the lumen due to peristalitic movements of the oviduct. When paraffin pellets were used, they were soon softened by body

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[1] We wish to acknowledge the help of Dr. H. W. Norton in the design of some of the experiments and their statistical analysis. We thank Dr. H. W. McShan and R. K. Meyer (Univ. of Wisconsin) for the ISAP, Mr. Irby Bunding of Armour and Co. of Chicago for the LH and Dr. R. E. Waterman of the Schering Corp., Bloomfield, N. J. for the progesterone.

heat, torn loose from the thread fastening them to the oviduct wall and passed out. It was thus noticed that the thread alone, which remained after the paraffin pellet was evacuated, provided sufficient stimulus to cause profound changes in the laying cycle of these hens.

ANIMALS AND TECHNIQUES

The birds used were sexually mature hens less than two years old, of the White Leghorn and New Hampshire breeds. They were trapnested prior to the experiments to determine their normal rate of production. Only regularly laying birds were used. They were maintained in individual cages in laying batteries and allowed free access to a standard laying mash and water.

At the time of operation the birds were anesthetized with nembutal, and the oviduct was exposed through an incision in the abdominal wall. A loop of coarse surgical thread was passed through the wall of the oviduct into the lumen of the magnum in such a manner as to extend longitudinally through approximately an inch of the lumen. The thread was again passed through the wall of the magnum and the two ends of the thread were tied externally. In placing the loop care was taken to assure that the lumen of the oviduct was not obstructed.

In a second group of birds the oviduct was exposed in the same manner but no loop of thread was placed in the oviduct (sham operated controls). Birds in both groups were weighed at the start and at the end of the experiment; their combs were measured on alternate days and individual daily records of egg production were kept.

The hormones used in some of the experiments included progesterone in oil; a partially purified gonadotrophic preparation containing both follicle stimulating (FSH) and luteinizing hormones (LH) called ISAP; and a highly purified preparation of LH. The gonadotrophic hormones were dissolved in water and injected intravenously. One single injection of ISAP was made while the LH was injected twice daily until ovulation occurred. The progesterone was injected subcutaneously. Control animals were injected with equal amounts of oil or water.

The laid egg was the criterion used for determination of ovulation, with the exception of one experiment involving ISAP in which the hens were sacrificed and the number of ovulations verified at autopsy. At the conclusion of some of the experiments the birds were sacrificed, the ovaries and oviducts removed, weighed, measured and described with regard to their physiological condition.

After the basic physiologic effects on the ovary and the oviduct of the operation described were established, the hens were no longer sacrificed since the laid egg was taken as the evidence for success of the treatment when hormones were injected.

RESULTS

In order to establish the quantitative and qualitative effects of the loop on the laying cycle of the birds, on their reproductive organs and their comb size, groups of birds were killed 5, 10, 15, 20, 25 and 30 days after the operation. Eight of the birds destined to be killed at the different intervals in each group had a loop in the magnum, and eight served as sham-operated controls. Due to mortality at operation and during the course of the experiment some of the groups did not contain the full complement of eight birds at the time of autopsy (Table 1).

The most striking effect of the loop in the magnum was that it almost completely inhibited ovulation during the first 25 days after the operation. There were a few sporadic ovulations which were widely spaced (Table 1). *Eight of the 33 birds with a thread in the magnum laid a total of 12 eggs* (1.5 eggs per bird). That the operation itself, in absence of the loop of thread in the magnum, is not responsible for the interruption of laying is seen from the fact that during the same period 29 sham-operated hens laid 121 eggs (5.5 eggs per bird).

TABLE 1. EFFECT OF A THREAD IN THE MAGNUM ON THE SIZE OF OVARIES, OVIDUCTS AND COMBS, AND THE RATE OF OVULATION. OBSERVATIONS MADE AT 5-DAY INTERVALS

Treatment	Days from operation to autopsy	Number of birds	Number of birds laying	Total number eggs laid	Ovary weight (gms.)	Oviduct weight (gms.)	Comb area (cm.)	Average size of follicles (cm.)	Average number of follicles of ovulatory size (30 mm.)
L	5	6	1	2	45	53	25	19	0.75
			p =	0.15*	SE 8.7	6.2	5.1	1.4	
S	5	5	3	7	48	55	33	22	0.40
					8.0	2.0	5.6	1.0	
L	10	8	2	4	48	53	23	21	0.40
			p =	0.05*	5.9	4.1	1.6	0.8	
S	10	7	5	12	51	63	23	23	0.6
					3.5	4.3	4.0	1.1	
L	15	5	1	1	56	65	27	22	1.0
			p =	0.001*	5.3	3.5	1.4	1.3	
S	15	7	7	44	58	67	32	23	1.0
					3.4	3.3	3.8	1.1	
L	20	7	2	2	68	68	31	24	1.71
			p =	0.05*	4.8	3.7	4.4	1.0	
S	20	5	3	24	52	47	24	22	1.25
					10.9	5.9	2.0	1.4	
L	25	7	2	3	17	42	29	all atretic	0.00
			p =	0.01*	2.5	3.9	2.3		
S	25	5	4	34	43	30	25	22	1.12
					10.4	13.5	3.6	1.5	
L	30	6	3	31	59	69	30	—	—
			p =	0.06*	6.8	8.6	1.4		
S	30	6	5	55	52	60	34	—	—
					6.4	4.4	3.8		

L—hens, with thread in magnum of oviduct.
S—sham operated controls.
*—*levels of significance of differences in egg production between S and L.*
SE—standard error of means.

In all subsequent experiments essentially the same results were found as those discussed here. The presence of an irritant in the magnum modified the laying cycle of all hens treated, although not all the animals treated responded by complete cessation of laying. In most hens, laying was suppressed completely (*in 75% of the hens in one experiment, in 58%* in another). Most remaining hens laid occasional eggs, but few continued to lay at an almost normal rate. In most cases the rate of production was reduced very drastically as compared to the almost unimpaired rate of production of the sham-operated group.

The second important observation regarding the presence of the thread in the oviduct was that 30 to 40 days after the operation laying was gradually resumed and eventually became normal (Table 1, 30-day group).

It is also interesting that the loop affected neither the size, the morphological condition of the ovary, nor that of the oviduct or the comb (Table 1). Ova which were present in the ovary at the time of the operation seem to be maintained without degenerating during the first 20 days after the operation, thus accounting for the non-reduction in ovarian weight (Table 1). This is true not only for number of follicles but also for size. In this particular experiment atresia of follicles set in at about 25 days and was observed in all seven hens in this group. At 30 days, when the next group was killed, the ovaries were found to be normal and three of the six hens in this group were laying. Statistical analysis showed that the differences in production between the sham-operated and the hens with a thread in the magnum were significant in all groups except the 5-day and the 30-day groups (Table 1).

These observations led to the assumption that the irritant in the oviduct did not depress pituitary function because the size of the ovary (number and size of follicles) was not reduced for as long as 20 days after the operation. Since neither the ovary nor the oviduct or the comb were reduced in size, it appeared that the ovary continued to secrete androgen and estrogen at normal rates.

These facts led to the theory that the presence of an irritant in the uterus specifically prevented the release of sufficient amounts of LH from the pituitary to cause ovulation without, however, interfering with the other endocrine functions of the pituitary. The mechanism of this inhibiting action on the secretion of LH was thought to be neuro humoral in nature and experiments were conducted to test this assumption.

In order to establish the relationship of the presence of the thread to the inhibition of ovulation, two experiments were designed. In the first, 12 hens which had ceased laying after the placing of the thread were injected intravenously with a crude gonadotrophic preparation, ISAP, containing

TABLE 2. NUMBER OF OVULATIONS OCCURRING WITHIN 72 HOURS AFTER ONE INTRAVENOUS INJECTION OF 0.25 GRAM EQUIVALENT OF ISAP (ISAP 16: 43.6 MGS. EQUIVALENT TO 1 GRAM OF ACETONE-DRIED SHEEP PITUITARY POWDER)

Results	Number of hens
Did not ovulate	2
Ovulated 1 ovum	4
Ovulated 2 ova	4
Ovulated 3 and 4 ova	2

both FSH and LH. The frequency of induced ovulations was very high, 10 of the 12 hens ovulating while none of the controls responded (Table 2). The two injected birds which did not ovulate were noted to be in poor physical condition at the time of autopsy. In this experiment the birds were killed 72 hours after the injection of the hormone and the number of ova shed was determined at autopsy (Huston and Nalbandov 1950).

TABLE 3. EFFECT OF INTRAVENOUS INJECTIONS OF LH (ARMOUR) ON OVULATIONS OF HENS WITH THREAD IN THE MAGNUM. (HENS INJECTED TWICE DAILY UNTIL FIRST OVULATION)

Treatment	Dose of LH (mgs.)	Number of hens not ovulating	Number of hens ovulating	Number of days from first injection to ovulation
Thread in magnum*	0.0	6	2	—
Thread in magnum*	0.1	1	6	2, 2, 3, 3, 4, 7
Thread in magnum*	0.2	3	5	2, 4, 5, 6, 8
Sham operated†	0.0	1	6	—

* Had not ovulated from operation to first injection (9 days).
† Were in normal production during the whole period.

Since six of the ten hens ovulating in response to the ISAP injection shed more than one yolk, the dosage used was thought to be unphysiologically high. In the second experiment LH (Armour) was injected at two different dosages into two groups of hens of seven and eight animals respectively with threads in their magna. Both dosages of LH were effective in causing ovulations in the majority of the hens although the number of injections required to cause ovulations was much greater than in the case of ISAP (Table 3). The dosages of LH seemed within the physiological range since no more than one ovulation was produced in any of the treated hens.

It has been shown that release of LH from the pituitary can be induced in the fowl by progesterone (Fraps and Drury, 1943; Rothchild and Fraps 1949). Accordingly, the ability of progesterone to cause ovulation in hens with threads in the oviduct was assayed in a series of tests involving dosages of progesterone ranging from 0.3 to 6.0 mgs. In all cases a single

TABLE 4. ABILITY OF VARIOUS DOSES OF PROGESTERONE TO CAUSE OVULATION IN HENS WITH THREAD IN THE MAGNUM

Dose of progesterone (mgs.)	Number of hens not ovulating	Number of hens ovulating	Average number of ovulations per hen	Number of days from injection to ovulation
(oil)	6	0	0	—
0.3	3	0	0	—
0.6	3	0	0	—
0.9	3	0	0	—
1.0	3	0	0	—
2.0	2	2	1.00	2, 3
4.0	3	2	1.50	2, 5
5.0	3	4	1.25	1, 2, 2, 5
6.0	1	3	2.00	1, 2, 2

subcutaneous injection of the hormone was given. The results (Table 4) indicate that it is possible to cause ovulation with progesterone in birds in which the release of endogenous LH has been blocked by the presence of a loop of thread in the magnum. Apparently the progesterone levels required for ovulation in these hens are higher than those which had been found effective by Rothchild and Fraps (1949). These authors had established the egg-laying pattern of each hen accurately enough to predict within a few hours the expected time of maturation of a follicle. In the present experiments no such determination could be made, since laying had been interrupted by the placing of the irritant and most of the follicles of ovulatory size were quite old when the progesterone injections were made. The reduced effectiveness of progesterone observed in our experiments may be due to a loss of sensitivity of the "aged" follicles to LH or a reduced sensitivity of the pituitary to progesterone. This may explain the failure to induce ovulation in nine out of twenty hens injected with two or more milligrams of progesterone (Table 4).

During the course of these experiments it was noted that 20 to 40 days after the thread had been placed in the magnum, most of the operated birds gradually began to lay and eventually returned to normal laying frequency. This has been explained on the assumption that the neural mechanism involved becomes conditioned to the constant irritation of the oviduct and loses its ability to respond to the stimulus. To test this possibility two experiments were performed. In one group of eight birds laying was stopped by the insertion of a thread in the magnum. When the thread was removed, seven of the eight birds began to lay normally within two weeks after the operation.

A second group of eight birds, which had a thread in the magnum was allowed to go through the usual non-laying period following the operation. As in earlier experiments these hens began to lay 30 to 40 days after the operation. As they returned into production a second operation was performed, the original thread was removed and a new one placed in the same general region of the magnum. Six of the eight hens subjected to this treatment continued to lay indicating that the oviduct as a whole loses its sensitivity to respond to the stimulus from the thread.

DISCUSSION

On the basis of these experiments it is suggested that a neural link exists between the oviduct and the pituitary gland of chickens. This is supported by the fact that the presence of an irritant in the magnum suppresses ovulation without affecting the other endocrine functions of the pituitary, the ovary or the responses of their respective target organs. It is known that comb growth in the hen is caused by androgen and that androgen secretion in turn is caused by LH stimulation of the interstitial cells of the ovary (Nalbandov, unpublished). Since in hens in which a thread was present in the magnum, comb-size remains unaffected (indicating continued secretion of *some* LH), the assumption becomes necessary that in the chicken ovulation is caused by the secretion of additional amounts of LH over and above that quantity of LH which is assumed to be produced at a constant rate. It is thought that the occurrence of these "ovulatory peaks" of LH are specifically prevented by the presence of an irritant in the magnum. This interpretation is supported by the observation that the injection of exogenous LH or of progesterone which releases endogenous LH from the pituitary, causes ovulation in hens with thread in the magnum, which stops as soon as injections are discontinued. This interpretation fits in with findings of Sawyer, Everett and Markee (1948, 1949) who showed that in the rat neurogenic activation of the pituitary is necessary for the release of LH. In the hen in which a thread is placed in the oviduct the neural pathway to the pituitary appears to be blocked.

It is of interest to note that in operated hens follicles which normally would have ovulated within a few hours, remained in a state of "suspended animation" over prolonged periods of time, in some cases for more than four weeks, without becoming atretic. Such follicles continued to secrete estrogen since the oviducts were maintained in size and retained their ability to secrete albumen. Furthermore, the follicles remained capable of ovulating over prolonged periods of time, as shown by the fact that ovulations resulted following injection of exogenous LH.

The interpretation presented fits most of the facts observed. There are certain findings which cannot be fitted into this scheme at the present time and which will require additional experimentation. Thus in some of the operated hens, particularly in the early experiments in which key-chains and other large irritants were used, the ovary enlarged considerably, in some cases containing six or more follicles of ovulatory size (30 or more mm. in diameter). This was interpreted to mean that the rate of secretion of FSH and possibily LH was increased appreciably by the presence of the irritant but here too ovulation was suppressed. In some hens the presence of a thread in the magnum did not suppress ovulation but on the contrary caused superovulation. Thus in one case four eggs were laid in two successive days although all of them were soft-shelled. In many other cases two soft-shelled eggs were laid in one day.

The presence of a uterine irritant produces a very high proportion of either soft-shelled eggs or yolks coated only with albumen or even yolks which are laid completely uncoated. In one experiment eight normal hens laid 63 eggs, only one of which was soft-shelled while eight operated hens ovulated with LH produced 16 eggs, eight of which were either soft-shelled or consisted of yolks coated with albumen only. One of these hens laid two eggs in 12 hours, the first egg with a normal shell, the second soft-shelled.

These facts show that uterine irritation may occasionally cause an increased rate of both FSH and LH secretion leading to superovulation.

The existence of this hitherto unsuspected neurogenic connection between the oviduct and the pituitary gland raises the question whether this relationship has any physiological significance in the normal hen or whether it is an artifact. It is thought that the mechanism described is physiologically important in that it may serve the hen as a timing device which prevents ovulation while a yolk (an irritant) is passing through the oviduct. Additional work will be needed before the details of such a signalling system are understood and can be fitted into the time schedule already worked out with regard to the release of LH and the length of time an egg normally spends in the oviduct and in the shell gland.

SUMMARY

The presence of an irritant, such as a loop of thread, in the magnum portion of the oviduct of laying hens, completely suppresses ovulation in the great majority of the treated animals. There is no reduction in the size of the ovary, the oviduct or the comb of these hens for as long as 25 days.

Injection of progesterone or of gonadotrophin containing LH into such hens causes ovulations but only so long as the injections are continued.

The presence of an irritant is thought to prevent the secretion of amounts of LH sufficient to cause ovulation (ovulatory peaks), but does not interfere with the secretion of levels of LH sufficient to assure normal androgen secretion, as is evidenced by the fact that in hens with a thread in the oviduct the comb does not decrease in size. The presence of the loop does not prevent the secretion of normal amounts of FSH since the ovary remains of normal size for as long as 25 days after the operation. For approximately the same length of time the ovary also contains follicles of ovulatory size (30 mm. or over) and in the same numbers as would be expected in unoperated hens. These follicles continue to secrete estrogen at normal levels since the oviduct in operated hens does not decrease in size and remains capable of secreting albumen.

This neurogenic system may be of physiological importance in the normal hen in that it may serve to synchronize ovulation with the other events of the laying cycle.

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THE POTENCY OF CERTAIN DESOXYCORTICOSTERONE ESTERS WITH RESPECT TO THE PRODUCTION OF HYPERTENSION AND CARDIOVASCULAR LESIONS*

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OF THE 11-desoxycorticoids which are available for therapeutic use, desoxycorticosterone acetate (DC-acetate) has enjoyed the widest acceptance. In an effort to increase the activity of the hormone either by conferring upon the steroid a more favorable absorption rate or reducing the rate at which it is metabolized by the body, various esters, in addition to the commonly used acetate, have been prepared. The water soluble DC-glucoside has proven to be very effective, when administered intravenously, in effecting the recovery of adrenalectomized dogs from the symptoms of acute adrenal insufficiency (Meier *et al.*, 1944; Swingle *et al.*, 1952). DC-benzoate, DC-palmitate and DC-butyrate have each been found, when given in identical dosage by single injections, to prolong the life of adrenalectomized rats more effectively than either dexoxycorticosterone or its acetate.

With the recent preparation of DC-trimethylacetate and DC-phenylacetate, attention has again been focused on the metabolic effects of desoxycorticosterone esters. Both of the latter compounds have been found to be more effective than the acetate when given as single injections, in maintaining the adrenalectomized dog free of insufficiency symptoms (Swingle *et al.*, 1952; Gross and Tschopp, 1952), or in prolonging the survival period of adrenalectomized rats (Gaunt *et al.*, 1952; Gross and Tschopp, 1952). Clinically, the trimethylacetate has been found, again administered as a single injection, to have prolonged effects in patients with Addison's disease and acute effects of overdosage have not been observed (Frawley and Forsham, 1951).

There is no reason *a priori* to suppose that the different esters of desoxycorticosterone would vary qualitatively in their actions, although quantitatively dissimilar activities might be anticipated. In view of the fact that toxic symptoms of overdosage with DC-acetate, including hypertension, edema and cardiac failure have been observed in man (Giraud

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et al., 1950; Willson *et al.*, 1940; Thorn *et al.*, 1940) and the rat (Selye and Hall, 1944; Friedman *et al.*, 1948), it might be expected that other desoxycorticosterone esters would display similar effects. Because the studies on DC-phenylacetate and DC-trimethylacetate reported to date appear to have been concerned exclusively with the effects of a single injection, an experiment designed to test the effect of chronic treatment with desoxycorticosterone esters in order to assess their relative toxicity with respect to the cardiovascular system appeared to be warranted.

METHODS

Forty female albino rats of the Holtzman strain weighing between 60 and 89 gms. with an average of 75 gms., were uninephrectomized and divided into four equal groups. The rats of groups 1, 2, and 3, each received 2 mg./day of DC-acetate,† DC-phenylacetate and DC-trimethylacetate respectively, by subcutaneous injection, in 0.2 cc. of the suspension medium. The animals of group 4 each received 0.2 cc./day of suspension medium and served as controls. All animals were maintained on a regimen of Purina Laboratory Chow and 0.9% NaCl solution *ad lib.*

Blood pressures, on unanaesthetized animals, were measured on the first day and once weekly thereafter until the experiment was terminated. A modification (Sobin, 1946) of the tail plethysmograph method (Williams *et al.*, 1939) was employed, and the arithmetic mean of four consecutive pressures agreeing to within 5 mm. Hg was taken to be representative of the blood pressure of the rat in question. The daily fluid intake of each of the groups of experimental animals was measured. The animals were sacrificed on the 28th day of the experiment, and the various tissues and organs were excised and placed in Bouin's solution for fixation and subsequent histologic treatment. After fixation, the organs to be weighed were transferred to water for 24 hours. They were then carefully dissected free of connective tissue, blotted on filter paper and weighed on a torsion balance.

RESULTS

Fluid Intake

The fluid intake of each of the four groups is given in Figure 1. It is apparent that the daily consumption of fluid was greatly augmented in each of the steroid treated groups, although those receiving DC-acetate showed the greatest evidence of polydipsia, and those receiving DC-trimethylacetate the least. Until the third week of treatment, at which time hypertension became more marked in the DC-trimethylacetate group, these animals were consuming about twice as much of the saline solution as controls, while those receiving DC-acetate consumed about three times as much. The fluid intake of rats receiving DC-phenylacetate, while slightly less than that of the DC-acetate group, paralleled the latter closely throughout the major part of the experiment.

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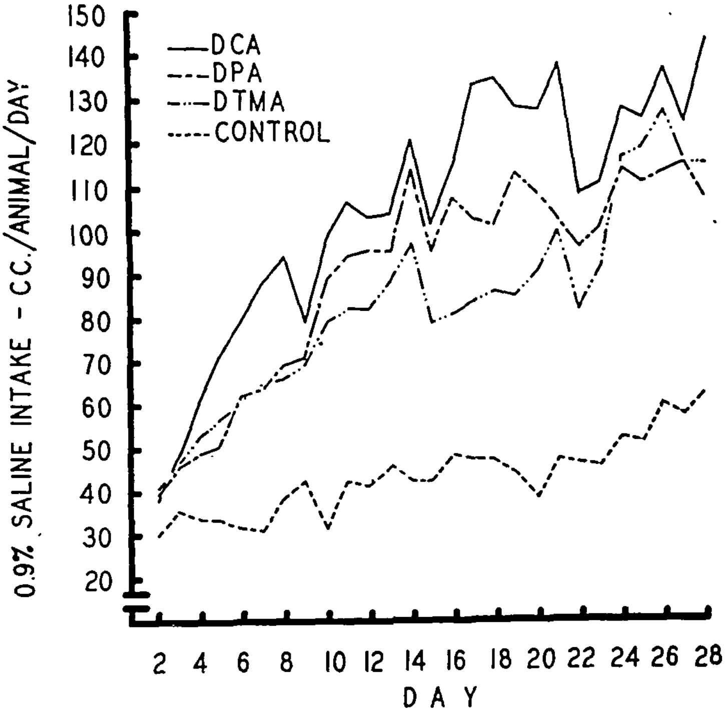

FIG. 1. Intake of 0.9% NaCl solution in control rats and rats receiving the acetate, phenylacetate and trimethylacetate esters of desoxycorticosterone.

Blood Pressure

In the various studies on hypertension which we have conducted in the rat, rarely is a pressure of 135 mm. Hg or higher encountered in unanesthetized normal animals from our colony. However, since an occasional rat may show pressures of 145 mm. Hg, a level of 150 mm. Hg has arbitrarily been selected as the lowest pressure consistent with a diagnosis of hypertension. While it is possible that a few animals having pressures which lie between 140 and 150 mm. Hg might be truly hypertensive while not considered as such, at least the level of 150 mm. Hg permits us to be more certain that such a pressure has resulted only through treatment.

No evidence of hypertension was encountered at any time in the control animals of group 4. The behavior of groups 1 and 2, receiving the acetate and phenylacetate respectively was essentially similar. There was early onset of hypertension, detected in the second week of treatment,which became progressively more severe, with the result that on the 26th day of treatment all of the animsls in these groups were hypertensive, the group average being in excess of 210 mm. Hg. The animals of group 3, receiving trimethylacetate, were much less affected. While there was an early onset of hypertension in four of the animals, i.e. on the 7th day, the incidence did not increase until the fourth week of treatment in which, for the first time, the average blood pressure of the group exceeded 150 mm. Hg. At the last blood pressure determination, the group average was less than 190 mm. Hg and two of the nine survivors were normotensive, having pressures below 140 mm. Hg. Hypertension in group 3 tended therefore, to be later in onset, milder in severity and of lesser incidence than in groups 1 and 2. Table 1 summarizes the data.

TABLE 1. EFFECT OF DESOXYCORTICOSTERONE ESTERS ON BLOOD PRESSURE OF THE RAT

Day	Blood pressure	Controls (group 4)	Desoxycorticosterone		
			Acetate (group 1)	Phenyl-acetate (group 2)	Trimethyl-acetate (group 3)
1	No. hypertensive	0	0	0	0
	Group average, mm. Hg.	112 ±2†	112 ±2	111 ±2	115 ±3
7	No. hypertensive	0	4	1	4
	Group average, mm. Hg.	111 ±2	142 ±6	135 ±4	148 ±4
14	No. hypertensive	0	5	7	3
	Group average, mm. Hg.	115 ±3	158 ±11	156 ±4	143 ±6
21	No. hypertensive	0	9	10	4
	Group average, mm. Hg.	115 ±2	176 ±11	193 ±7	147 ±10
26*	No. hypertensive	0	9	9	7
	Group average, mm. Hg.	119 ±2	218 ±11	214 ±10	183 ±12

* Nine animals surviving in each steroid treated group on this day.
† Mean ±S.E.

Miscellaneous Observations

The animals of groups 1 and 2, which showed early onset of severe hypertension and marked polydipsia also developed edema and ascites during the second week of treatment and continued to manifest these changes in varying degree throughout the period of treatment. The animals of group 3, treated with DC-trimethylacetate, increased their fluid intake markedly, i.e. from about 90 cc./rat/day to about 125 cc./rat/day during the last week of treatment, at this time they also began to develop the edema and ascites which had an earlier onset in groups 1 and 2, and an additional three animals became hypertensive.

Retinal hemorrhages, a not uncommon symptom of hypertensive oculopathy in DCA treated rats, was observed in two animals in group 1 and one animal in group 2. They did not occur in groups 3 and 4.

Organ Weights: These data are given in Table 2.

Adrenals. There was no significant alteration in adrenal weight as a result of treatment with the DC-esters. This is in conformity with published observations on the effect of DC-acetate. Since the main effect of 11-desoxycorticoids is to cause involution of the cortical zona glomerulosa, which constitutes an insignificant proportion of the total bulk of the gland, changes in adrenal weight are never pronounced in animals treated with these hormones.

Kidneys. The renal enlargement, so characteristic of DCA treated rats, was equally marked in rats treated with DC-phenylacetate and DC-trimethylacetate; there being no significant difference in renal weight between any of the steroid treated groups.

TABLE 2. ORGAN WEIGHTS AND CARDIOVASCULAR LESIONS IN CONTROL AND STEROID TREATED RATS

Item	Controls	Desoxycorticosterone		
		Acetate	Phenylacetate	Trimethylacetate
Body wt. g. Initial Final	 73 ± 3 146 ± 17	 75 ± 3 141 ± 5	 75 ± 4 122 ± 6	 75 ± 3 141 ± 4
Adrenal mg./100 g.	24.6 ± 1.5	22.0 ± 0.9	29.0 ± 2.0	23.7 ± 1.4
Heart mg./100 g. Incidence and severity of lesions	298 ± 14 0 (10)	449 ± 43 + (3)* ++ (1) +++ (2) 0 (3)	453 ± 29 + (2) ++ (3) +++ (3) 0 (1)	378 ± 27 + (4) ++ (1) +++ (0) 0 (4)
Kidney mg./100 g. Incidence and severity of lesions	621 ± 15 0 (10)	1307 ± 89 + (3) ++ (4) +++ (2) 0 (0)	1360 ± 96 + (2) ++ (3) +++ (4) 0 (0)	1118 ± 104 + (2) ++ (3) +++ (2) 0 (2)
Pancreas Incidence and severity of lesions	0 (10)	+ (5) ++ (1) +++ (0) 0 (3)	+ (3) ++ (2) +++ (3) 0 (1)	+ (0) ++ (3) +++ (0) 0 (6)

* Severity, designated by pluses, is followed by bracketed figure indicating incidence.

Heart. Cardiac hypertrophy was evident in each of the steroid treated groups, although not as marked in the group which received DC-trimethylacetate as in those treated with the acetate or phenylacetate.

Cardiovascular Lesions

Histologic examination of the kidney, heart and pancreas, the latter having proven to be a site of predilection for the necrotizing arteriolitis which occurs in hypertensive DCA treated rats, revealed that typical lesions were present in animals in each of the groups. However they were of lower incidence and lesser severity in the trimethylacetate than in the acetate or phenylacetate treated rats. A summary given in Table 2 synopsizes the data. The severity of lesions are graded on a scale of 0 to +++. In the pancreas the lesions were entirely vascular, of the periarteritic type; in the heart they consisted of either arteriolar necrosis with an associated perivascular accumulation of inflammatory cells, resembling the "Aschoff nodule"; or as an interstitial myocarditis. The renal lesions were those of arteriolosclerosis and necrosis, glomerulosclerosis and occasionally focal infiltrations of inflammatory cells. The incidence of lesions in each of these tissues was approximately equal in the acetate and phenylacetate groups and less in the trimethylacetate treated rats.

DISCUSSION

Insofar as polydipsia and the development of hypertension may serve as criteria in the estimation of mineralocorticoid toxicity, these experiments suggest that DC-acetate and DC-phenylacetate are of approximately equal potency whereas DC-trimethylacetate is somewhat weaker. The conclusion is also supported by the incidence and severity of cardiovascular lesions. Although, it would be hazardous to draw sweeping conclusions from one experiment involving so few animals, in view of the variability of such responses (Rosenberg *et al.*, 1952), the present experiment suggests that DC-phenylacetate is somewhat more toxic than DC-acetate.

The effects of DC-trimethylacetate, as compared with those of the acetate and phenylacetate derivatives, were relatively delayed in onset. This was manifest in saline intake and the development of hypertension. Since we have never observed lesions of the type described in desoxycorticosterone-treated rats unless hypertension was present, and since the severity of the lesions is correlated with the duration and severity of hypertension, it was not surprising that the cardiovascular lesions were of lower incidence and lesser severity in the DC-trimethylacetate group.

The conclusion which appears to be best compatible with the results obtained is that each of the esters is capable of producing hypertension and cardiovascular damage, but that DC-trimethylacetate, possibily because of a slower absorption rate, requires a longer period of time to exhibit the same toxic potentiality as the acetate and phenylacetate.

Since the evidence previously cited indicates that at a given dosage DC-trimethylacetate exerts a more prolonged effect than the acetate, and inasmuch as it is more effective than DCA even at a lower dosage in supporting life and growth in the adrenalectomized rat (Gaunt *et al.*, 1952), it would seem likely that DC-trimethylacetate will prove, because of its lower toxicity, to be preferable to the acetate in controlling the electrolyte imbalance in patients.

SUMMARY

A comparison of the toxicity of three desoxycorticosterone esters, namely the acetate, phenylacetate and trimethylacetate, has been made in the rat. The criteria used were the induction of hypertension and cardiovascular lesions in the uninephrectomized rat maintained on a high NaCl intake and receiving 2 mg./day of the steroid subcutaneously.

The results indicate that the onset of hypertension, severe polydipsia, edema and ascites is rapid in rats receiving either the acetate or phenylacetate and delayed in rats treated with the trimethylacetate ester.

Cardiovascular lesions, while present in all groups were of lowest incidence and least severity in rats treated with DC-trimethylacetate.

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A QUANTITATIVE STUDY OF THE VASCULARITY OF THE ADRENAL GLAND OF THE RAT DURING PREGNANCY AND AFTER CORTISONE TREATMENT[1,2]

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THE maintenance and activity of cells of an organ is dependent among other things on the functional adequacy of the blood supply. The functional adequacy of the circulation of any organ depends in turn on a number of factors: the blood volume, rate of blood flow, the concentration of gases, nutrients and metabolites in the blood and tissue fluid, the tissue permeability of these substances, and the area of the capillary bed. In an endocrine organ, like the adrenal cortex, these factors are especially important, as the functional activity is expressed by the pattern of blood circulation. The complexities in the morphology of the adrenal cortex of mammals are so great that it seems practical at the present time to approach experimentally only the estimation of the area of the capillary bed. This area may be expressed as a ratio of the surface of the capillary bed to the volume of tissue occupied by it (S. V.).

There are no quantitative estimations of the vascularity in the adrenal gland despite several good qualitative approximations (Sjöstrand, 1934; Gersh and Grollman, 1941; Bennett and Kilham, 1940). The difficulties in the way of a quantitative estimation are apparent. It was hoped that many of these difficulties, if not most, could be avoided by the use of fixation by freezing and drying. Such a study has been made by Gersh and Still (1945) for fat tissue, and essentially the same technique was used in this study.

The experiments were undertaken in normal female and pregnant rats, with and without cortisone treatment. After very rapid fixation of the adrenals in situ by freezing with flowing isopentane at about −150° C., blocks of tissue were removed and dried at a low temperature. They were

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embedded in celloidin and sectioned serially. Camera lucida drawings of the outlines of the capillaries containing red blood cells were made. From the drawings were obtained data that gave the ratio of the total capillary surface to the volume of tissue supplied by the functioning vessels (S/V ratio), and the mean diameters of the capillaries.

It was found that marked changes of the S/V ratio and of the mean diameter of the capillaries occurred in the zona fasciculata and in the zona reticularis during pregnancy and after cortisone acetate treatment. In marked contrast is the relative constancy of the vessels in the zona glomerulosa. The changes in the medullary capillaries, if significant, were small.

MATERIAL AND METHODS

Preparatory phase. Twelve female rats of the Sprague-Dawley strain were used. They were fed "Complete Rockland Rat Diet" and they were allowed water *ad libitum.* Three normal rats served as controls; 3 non-pregnant rats were treated with 3 mg. cortisone acetate daily for 19 days. Six animals in proestrus were caged with males in the afternoon and when sperm were identified in the vaginal smear on the following morning this was considered as day one of pregnancy. Three of these rats were treated with 3 mg. cortisone acetate daily for 18 days; the other 3 received no treatment. Table 1 presents the summary of the weights of the animals at the beginning and at the end of the preparatory phase.

TABLE 1

No.		Weight at the beginning, gms.	Weight after 18–19 days, gms.
1		192	221
2	Controls	196	219
3		188	217
4	Non-pregnant rats. Daily 3 mg. Cortisone acetate	183	148
5		196	165
6		182	156
7		187	280
8	Pregnant rats	185	269
9		188	267
10	Pregnant rats 3 mg. Cortisone acetate daily	193	240
11		196	251
12		201	251

Summary of the weights of the animals at the beginning and at the end of the preparatory phase.

Preparation of the adrenals. Under light ether anesthesia the abdominal cavity was rapidly opened, the left adrenal exposed and immediately frozen in situ by a stream of isopentane chilled to about −150° C. The region including the frozen adrenal was then broken out with a chilled bone forceps. The gland was transferred to the drying chamber, and dried at −25° C. for eight days (Gersh, 1932). The adrenals were then placed in absolute alcohol at −25° C., and after 24 hours allowed to warm up to room temperature.

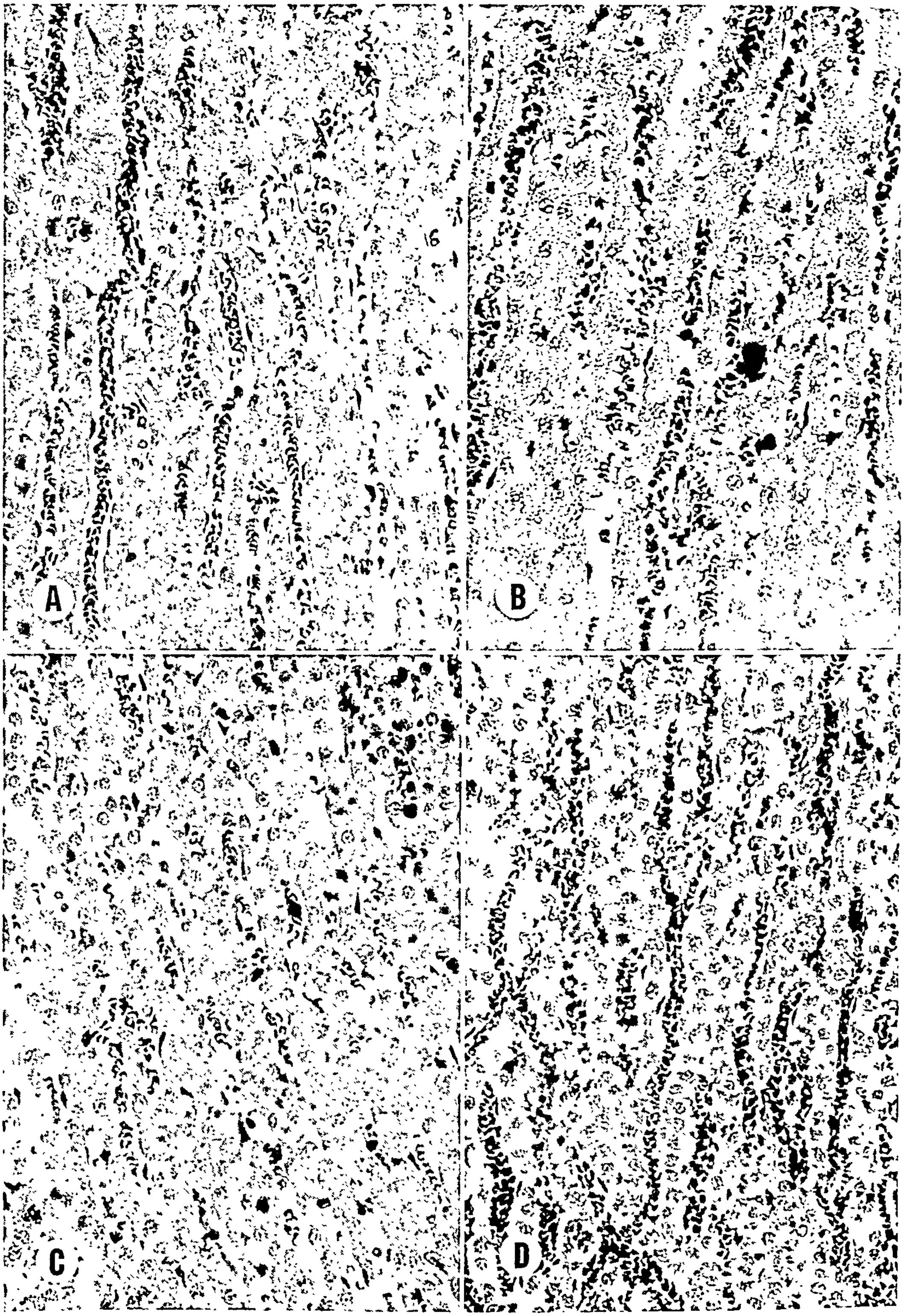

Plate I

TABLE 3

		Controls	3 mg. cortisone acetate daily	Pregnant rats	Pregnant rats 3 mg. cortisone acetate daily
Zona glomerulosa	Width	68.7	90.0	64.2	107.3
	Volume mm.^{3}	3.4	2.8	3.3	3.1
	Total capillary surface mm.^{2}	1176	1056	1003	1062
Zona fasciculata	Width	833.6	529.0	817.0	585.0
	Volume mm.^{3}	25.8	9.1	24.6	10.4
	Total capillary surface mm.^{2}	20744	3365	31857	2552
Zona reticularis	Width	401.8	242.7	370.5	283.5
	Volume mm.^{3}	4.5	1.8	3.9	2.0
	Total capillary surface mm.^{2}	5115	1523	5846	1168
Medulla	Width	703.3	656.9	737.5	597.5
	Volume mm.^{3}	1.6	1.2	2.2	0.9

The width, volume and total capillary surface of the different zones under the different experimental conditions. Marked changes in the total capillary surface in the zona fasciculata and zona reticularis are in contrast to the relative constancy of the vessels in the zona glomerulosa. The total surface of functioning capillaries of the medulla was not determined because measurements of the medullary sinusoids and veins were not made.

the pregnant rats the S/V ratio was significantly lowered and the mean diameter of the capillaries was reduced below that of untreated controls (3.6 microns for both treated groups) (Fig. 2 A, B, C, D.).

These differences in the surface area of capillaries per unit volume are magnified when one takes into account changes in the volume of the fascicular zone (see Table 3). The total capillary surface of the zone showed an increase from 20,747 mm.^{2} to 31,857 mm.^{2} in pregnancy and a decrease to 3,365 mm.^{2} and 2,552 mm.^{2}, respectively after cortisone treatment.

Zona reticularis. There was a definite increase of the S/V ratio during pregnancy and possibly an increase of the mean diameter of the capillaries

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EXPLANATION OF PLATE I

FIG. 2. Photomicrographs to illustrate changes in the diameter and in the number of open capillaries of the zona fasciculata of the rat are more numerous and their lumens are wider in diameter than in the non-pregnant rat. After treatment with cortisone the diameter and the number of open capillaries were markedly reduced in both pregnant and non-pregnant animals. The adrenals were prepared by freezing and drying, embedding in celoidin, and staining with hematoxylin eosin (×550).

A. Normal, non-pregnant. B. Normal, pregnant. C. Non-pregnant, treated with cortisone, and D, pregnant, treated with cortisone.

(from 9.3 microns for normals to 10.0 microns for pregnant animals). (See Table 2.) After cortisone treatment of non-pregnant and pregnant rats the S/V ratios were reduced below figures obtained in the untreated controls. The mean diameter in the non-pregnant group was reduced to 6.4 microns and in the pregnant group to 6.7 microns after cortisone administration.

The total capillary surface in the zona reticularis was increased during pregnancy (5,115 mm.^{2} for normal rats, and 5,846 mm.^{2} for pregnant rats). Cortisone acetate treatment caused a definite decrease of the total capillary surface of the reticular zone in non-pregnant rats (1,523 mm.^{2}) as well as in pregnant animals (1,168 mm.^{2}).

Medulla. A slight decrease of the S/V ratio which may be not significant was observed after cortisone treatment. The mean diameter of the capillaries was reduced from 8.5 microns (non-pregnant) and 7.5 microns (pregnant) to 6.2 microns and 6.0 microns, respectively; whether these changes are significant is also not known. No changes occurred in the pregnant rats. The total capillary surface was not determined in the medulla because measurements of the medullary sinusoids and veins were not taken. As these constitute a significant proportion of the total medullary volume the values of the total capillary surface in the absolute volume of the medulla would be misleading.

DISCUSSION

The very rapid fixation of the adrenals in situ by freezing at about −150° C. makes it possible to determine approximately the dimensions of the capillaries containing red blood cells at the moment of freezing (Gersh and Still, 1945). The knowledge of the so obtained S/V ratio of the different zones of the adrenal contributes to the understanding of the functional activity of the gland.

In contrast to findings in other species several investigators (Donaldson, 1924, 1928; Anderson and Kennedy, 1933; Blumenfeld, 1934) did not find a significant increase in adrenal weight during preganncy. However, a definite increase of the S/V ratio and of the total capillary bed of the zona fasciculata and zona reticularis was observed during pregnancy in these experiments. The S/V ratio for the zona reticularis of the pregnant rats (1,483) is the highest S/V value ever reported for tissue. Catchpole and Gersh (1947) calculated for the medulla of the mouse kidney 1,400, for the kidney cortex 850, for the heart muscle of man 1,184, for the mouse liver, 800. The values for other tissues (skeletal muscle, brain cortex, cerebellum, duodenum, pancreas) range between 190 and 500.

Severe adrenal atrophy in rats has been described after high dosages of cortisone (Ingle and Mason, 1938; Wells and Kendall, 1940; Stebbins, 1950; Winter *et al.*, 1950) due to reduction in size of the cells of the fascicular and reticular zones. A complete loss of lipid from the cells of these zones has been observed. Sourkes and Henage (1951) stated that adrenals of cortisone treated rats showed a decreased endogenous respiration as well as greatly reduced rates of oxidation of various intermediates of the Krebs' cycle. While the total volumes of the zona fasciculata and zona reticularis were reduced to nearly one third of the original volumes after cortisone acetate treatment, the total capillary surface of these zones was reduced to approximately one fifth to one tenth of the capillary bed of the controls and of the pregnant adrenals.

Sjöstrand (1934) stated that the same factors which are responsible for excessive cortical activity are effective on the vascular pattern of the medulla. Gersh and Grollman (1941) reported that the same vascular adjustments appear to take place in the medulla during hyperactivity as were described in the cortex, though to a less marked degree. It seems that no change takes place in the medulla of the adrenals of pregnant rats. The decrease in the S/V ratio and in the mean diameter of the capillaries was so small after cortisone treatment that these changes may be considered also as not significant.

SUMMARY

The ratio of the surface of the capillaries to the volume of tissue supplied by vessels (S/V ratio) of the zona fasciculata was markedly increased in pregnancy, and reduced below normal levels after the daily administration of 3 mg. cortisone acetate for a period of 18–19 days. Similar changes of the S/V ratio were obtained in the zona reticularis.

These differences in the surface area of capillaries became magnified by taking into account the volume of the zones. The total capillary surface of the zona fasciculata was increased during pregnancy by one half. It was reduced by one fifth to one tenth after cortisone acetate treatment.

Changes of the vascularity of the zona glomerulosa were not observed under the different experimental conditions. The S/V ratio in the medulla was not altered markedly in the pregnant animals and the slight decrease of the S/V values after cortisone treatment was too small to be considered significant.

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EFFECTS OF TESTOSTERONE PROPIONATE ON HISTOCHEMICAL REACTIONS OF RAT SEMINAL VESICLES

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INTRODUCTION

THE English anatomist, John Hunter (1786), was the first to describe the seminal vesicles of the rat. His description is as follows: "the bags are large and flat with serrated edges and lie someway within the abdomen, containing a thick ash coloured mucus, nearly the consistency of soft cheese. . . ." Nearly 150 years later Moore, Hughes, and Gallagher (1930) made a detailed cytological study of this organ and demonstrated the fundamental relationship existing between the testicular hormone and the functional activity of the secretory epithelium. Recently Porter and Melampy (1952) have presented the results of a biochemical study of the composition and metabolism of this gland.

This investigation deals with the effects of testosterone propionate on the structure and function of the seminal vesicles of the rat. Histochemical observations were made on basophilia, the localization of alkaline and acid phosphatases, and periodic acid-reactive carbohydrates. Furthermore, information is presented on the distribution of connective tissue in the gland as well as mitotic activity. Intact, castrate, and hormone-treated castrate animals were used in this work.

MATERIALS AND METHODS

Histochemical changes in the seminal vesicles of the rat were studied in 3 groups of animals: (1) 6 intact sexually mature males, (2) 19 castrates, 2, 5, 10 and 20 days after gonadectomy, and (3) 23 castrates receiving subcutaneously 500 μg. of testosterone propionate (T.P.)† daily for 2, 5, 10, and 20 days. The seminal vesicles of the animals in group 3 were allowed to atrophy for a 20-day period following castration prior to hormone treatment. The animals in groups 2 and 3 were mature males weighing 240–255

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† Peranderen, Ciba Pharmaceutical Products, Inc., Summit, N. J.

ble sources of error in the use of purified ribonuclease in cytochemical studies have been discussed by Stowell and Zorzoli (1947).

The lamina propria contained methylene blue-staining cells, pink fibers, and brightly stained erythrocytes and no basophilia was observed in the arterioles and venules. The secreted material present in the lumen was stained with eosin.

Alkaline and acid phosphatases. The results obtained by a histochemical investigation of the influence of male sex hormone on the alkaline and acid phosphatase activity of the seminal vesicles of the rat as determined by the method of Goetsch, Reynolds, and Bunting (1952) are presented in Tables 1 and 2 and Figures 5, 6, 7, and 8. A gradient of apparent alkaline phosphatase reaction occurred adjacent to the interacinar stroma in sections deparaffinized prior to incubation which makes critical evaluation of the localization difficult. Furthermore, the acid phosphatase reaction in deparaffinized sections was somewhat variable. Incubation of non-deparaffinized tissue sections gave a more accurate localization of enzyme activity. No alkaline or acid phosphatase activity was found in air-dried smears of seminal vesicle secretion. These results confirm chemical

TABLE 1. ALKALINE PHOSPHATASE LOCALIZATION IN SEMINAL VESICLES OF INTACT, CASTRATE AND HORMONE-TREATED CASTRATE RATS

	Intact animal	10-day castrate	20-day castrate	5-day injected	10-day injected	20-day injected
Epithelium						
A. Nucleus	0*	0	0	0	0	0
B. Cytoplasm	0	0	0	0	0	0
C. Secretion granules	0	0	0	0	0	0
Connective tissue (lamina propria)	+++	+++	++	++++	+++	+
Blood vessels in lamina propria	+++	+++	++	++++	+++	+
Smooth muscle	+	0	0	0	0	0
Blood vessels in smooth muscle	+	++	+	+++	+	+

* Evaluation: ++++, intense; +++, strong; ++, weak; +, faint; 0, no reaction.

EXPLANATION OF PLATE I

FIG. 1. Basophilia in seminal vesicles of intact animal. Toluidine blue. ×160.

FIG. 2. Same as Fig. 1. Following ribonuclease prior to toluidine blue. ×160.

FIG. 3. Basophilia in seminal vesicles of intact animal. Eosin-methylene blue. ×500.

FIG. 4. Seminal vesicles of 20-day castrate. Atrophied secretory epithelium. Eosin-methylene blue. ×500.

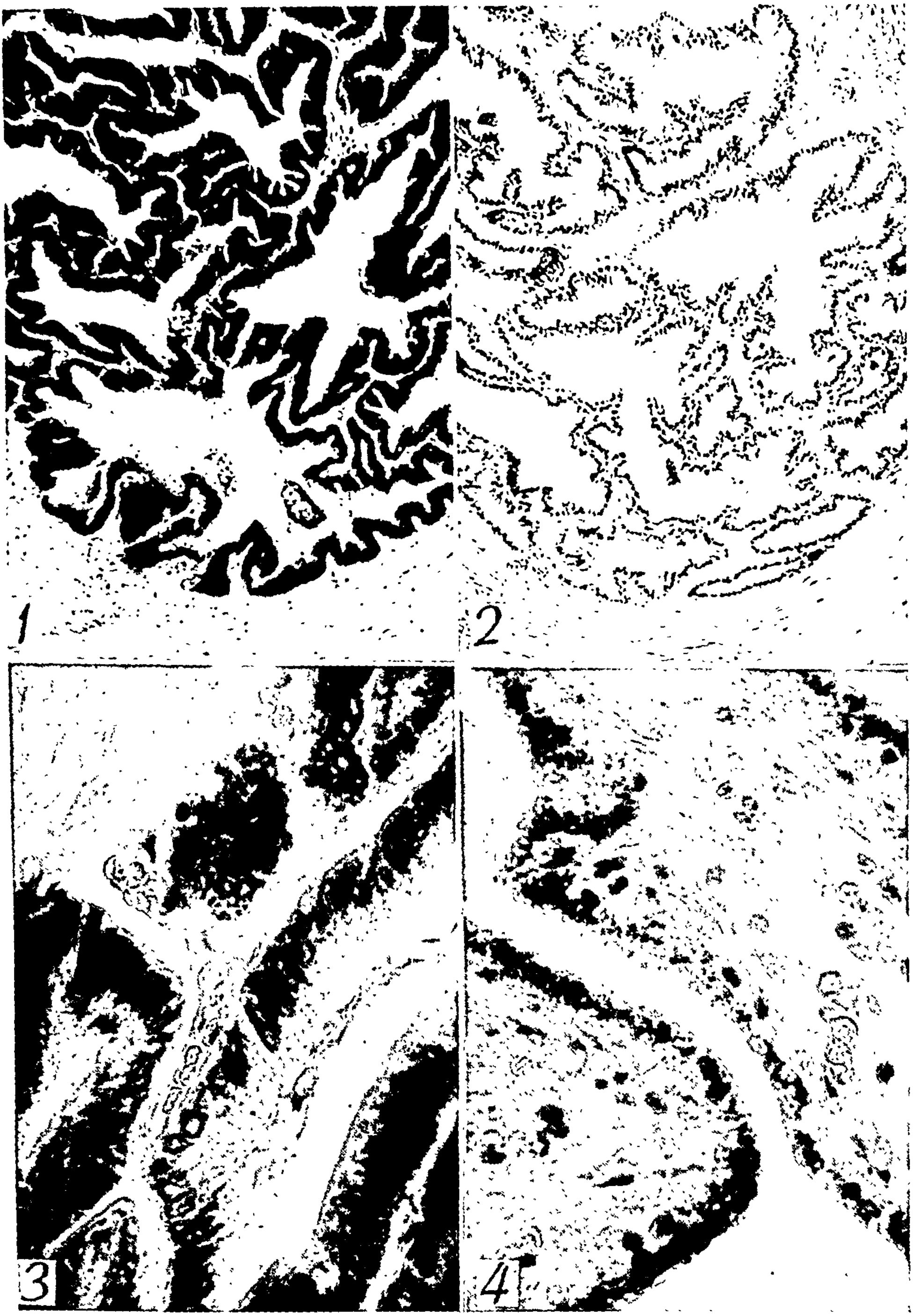


PLATE I

analyses which indicated the absence of both enzymes in the secretion of the rat.

Connective tissue. The outer layer of the seminal vesicles of intact animals was composed of a fine, thin capsule of connective tissue; whereas the smooth muscle zone surrounding the lamina propria (interacinar stroma) was relatively thick and was interwoven with elastic tissue. The lamina propria was formed chiefly of elastic fibers, and numerous arterioles and venules were widely distributed throughout this structure. No basement membrane was visible as such and the epithelium was seen to rest directly on the connective tissue as previously described by Moore, Hughes, and Gallagher (1930). The lamina propria divided into secondary and tertiary

TABLE 2. ACID PHOSPHATASE LOCALIZATION IN SEMINAL VESICLES OF INTACT, CASTRATE AND HORMONE-TREATED CASTRATE RATS

	Intact animal	10-day castrate	20-day castrate	5-day injected	10-day injected	20-day injected
Epithelium						
A. Nucleus	+++*	++	+	+++	+++	+++
B. Cytoplasm	+++	†	†	+++	+++	+++
C. Secretion granules	+++	0	0	++++	++++	++++
Connective tissue (lamina propria)	+++	++	+	++++	++++	++++
Blood vessels in lamina propria	+++	++	+ or ++	++++	++++	++++
Smooth muscle	++	+	+	+++	+++	+++
Blood vessels in smooth muscle	++	++	+ or ++	+++ or ++++	+++	+++

* Evaluation: ++++, intense; +++, strong; ++, weak; +, faint; 0, no reaction.

† Reaction appeared to decrease but because of the small amount of cytoplasm present in each cell it was difficult to evaluate the intensity of the reaction.

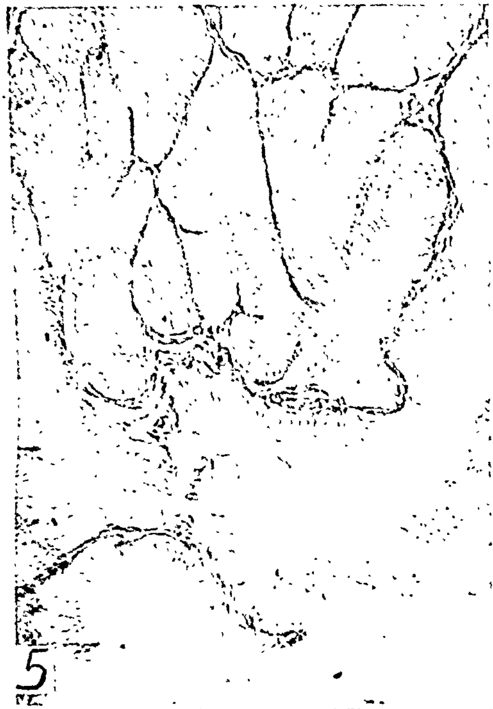
EXPLANATION OF PLATE II

FIG. 5. Alkaline phosphatase localization in connective tissue of seminal vesicles of intact animal. No reaction in epithelium and secretion in lumen. Two hours' incubation. ×160.

FIG. 6. Alkaline phosphatase in the seminal vesicles of a 5-day T. P. injected castrate. A stronger reaction was obtained in the connective tissue of this stage than in Fig. 5. Two hours' incubation. ×160.

FIG. 7. Seminal vesicle tissue of a 20-day castrate showing acid phosphatase localization. Four hours' incubation. ×160.

FIG. 8. Acid phosphatase in the seminal vesicles of a 5-day T.P. treated castrate. Intense activity at the apical end of the epithelium. Four hours' incubation. ×160.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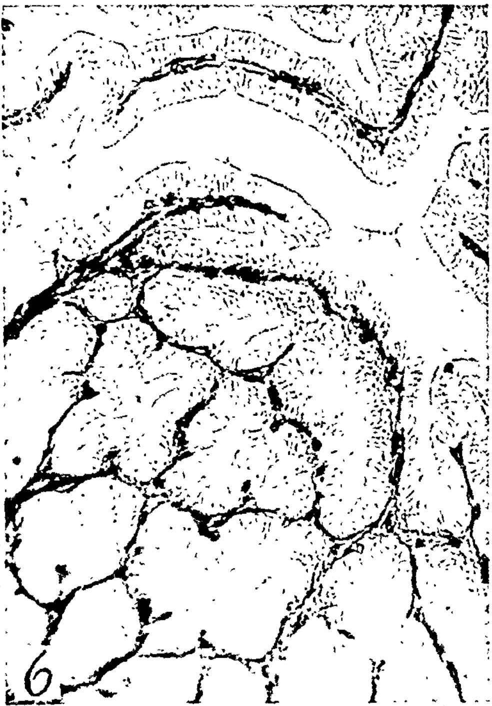
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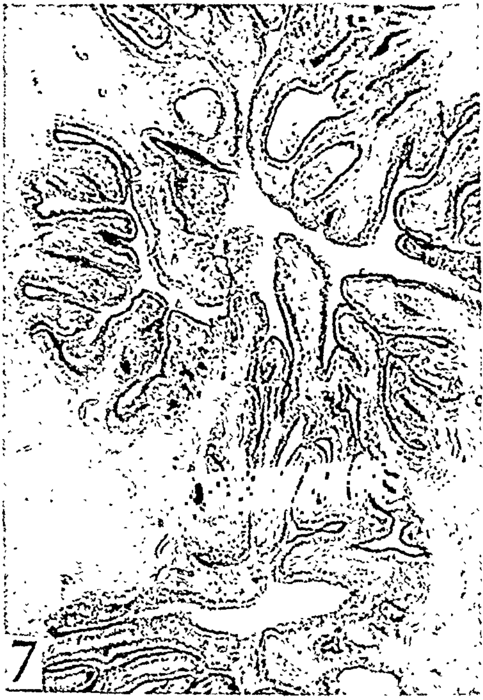
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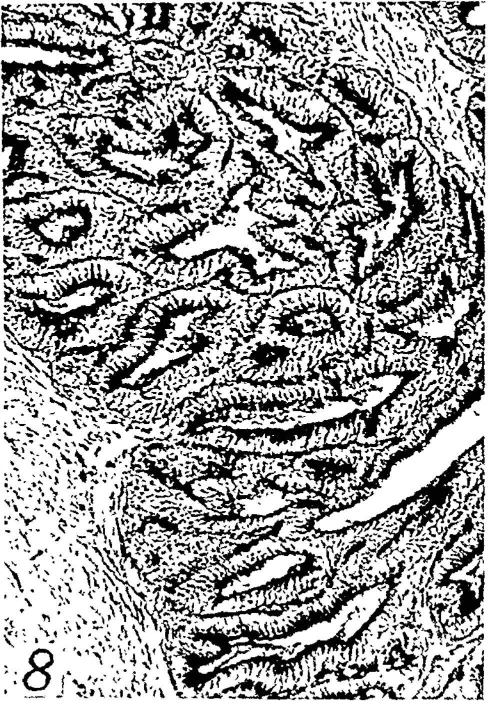
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PLATE II

DISCUSSION

In a biochemical investigation of the seminal vesicles of the rat (Porter and Melampy, 1952) it has been demonstrated that protein synthesis is an important function of this organ and is androgen controlled. The secretion produced by this organ in normal males contained 13.6% total nitrogen on a dry-weight basis. Castration of mature rats caused a decrease of 73% in the fresh weight of the secretion-free glands after 20 days and there was a 74% decrease in the total nitrogen content of this organ in the same interval. The catheptic activity decreased to 0.9 of a unit after 24 days in contrast to 2.8 units at the time of gonadectomy. Two days following castration there was a 78% decrease in the quantity of secretion initially present in the glands. The secretion had decreased 98% by the fifth day, 99% by the tenth day and was completely absent by the twentieth day. Subcutaneous injection of testosterone propionate (500 μg.) per day into castrate rats for 20 days resulted in the following increases per gland: wet weight 48 to 525 mg., nitrogen 0.2 to 7.3 mg. and the amount of secretion 0 to 1322 mg. The catheptic activity per gland of rats that were injected over a 10-day period increased from 0.9 to 4.5 units during this time.

In the present study it has been possible to extend these observations on the metabolism of the seminal vesicles by the application of histochemical methods for basophilia using toluidine blue and eosin-methylene blue. The presence of ribonucleoprotein in the secretory epithelium of this organ is suggested because the cytoplasmic and in many cases nucleolar basophilic reactions are abolished by treating sections with ribonuclease. This basophilia indicates an accumulation of ribonucleoprotein which may be related to the endocellular protein-synthesizing mechanism which is functional in this organ of the sexually mature rat. It is of interest that Wislocki (1949) has reported that cytoplasmic basophilia was absent from the epithelium of deer seminal vesicles but the cytoplasm had a strong affinity for acid dyes.

Recent investigations have shown a correlation between the nucleic acid content of cells of various tissues and their activity in protein synthesis. For example, in the pancreas, Caspersson (1950) has demonstrated that there is an intense protein metabolism and that the nucleotides of the nucleoli and cytoplasm are conspicuous. After pilocarpine stimulation there is a rapid synthesis of protein by the acinar cells which is preceded by nucleolar growth. Another example of this relationship is found in the gastric mucosa in which the protein-producing cells are characterized by a strong reaction for ribose nucleotides when compared to the parietal cells.

Deane (1946) in a study of the basophilia of hepatic cells of mice has demonstrated that the degree of the reaction is related to the metabolic activities of the organ. For example, *in the livers of mice killed after a* known feeding time, changes in the amount and distribution of basophilic material occurred. Dempsey and Wislocki (1945) have shown that basophilia is a characteristic feature of the cytoplasm of the syncytium and cytotrophoblast of the human placenta. This reaction in the syncytium was most marked in early pregnancy and disappeared during the last months of gestation. Ribonucleoprotein was also reported as a component of the syncytial trophoblast of the cat and the trophoblast of the sow by Wislocki and Dempsey (1946 a, b). In an investigation of the histochemistry of the rodent's placenta Wislocki, Deane, and Dempsey (1946) observed cytoplasmic basophilia in the labyrinth and yolk sac of the mouse, rat, guinea pig, rabbit and hamster. It decreased gradually in the labyrinth but persisted unaltered in the yolk sac until term.

Rabinovitch, Junqueira, and Rothschild (1951) reported that testosterone propionate increased the amount of ribonucleic acid phosphorus of seminal vesicles of castrate rats. These investigators indicated that their findings support the suggestion of Caspersson (1950) dealing with the relation between ribonucleic acid and protein synthesis.

Lesher (1951) has observed that in the case of the salivary gland of Drosophila larvae there appears to be a close correlation between cytoplasmic basophilia and nucleolar size.

In the present study the nuclei in the secretory epithelium of the seminal vesicles of intact animals were large and elliptical and nucleoli were visible in most of them. Two days after gonadectomy a few of the nuclei were pyknotic and contained small nucleoli. Twenty days following castration many nuclei were pyknotic and in numerous cases the nucleoli were absent or greatly reduced in size. However, in some instances, these structures were similar to those observed in intact animals and this variation may be due to differences in cellular activity. After 20 days of hormone injection, both nuclei and nucleoli of the secretory epithelium resembled those described as characteristic for intact animals. The changes observed in the nucleolus suggest that this structure is of importance in the secretory activity of the epithelium of the seminal vesicles.

Alkaline phosphatase activity was absent from the nuclei, cytoplasm and secretory granules of the epithelium of seminal vesicles of intact animals as well as castrates and hormone-treated castrates (Table 1 and Fig. 5). The connective tissue, particularly the lamina propria and its associated blood vessels, gave a strong reaction which became progressively weaker 10 days after castration. However, after 5 days of hormone injection, the reaction of this tissue became intense but after this time this reaction decreased as is shown in Table 1. This apparent decrease in the histochemical reaction may be related in part to the great enlargement of the acini at this time and as a result the stroma became stretched and relatively thin.

Alkaline phosphatase concentration as measured by its activity per gm. dry weight of secretion-free tissue varied during the 20 days of hormone treatment; this activity was greatest by the fifth day when the average wet weight of the gland was 203 mg., and after this time there was a decrease in activity which was associated with the increase in the size of the gland which was 525 mg. after 20 days of injection (Porter and Melampy, 1952). Due to the greater relative increase in the various constituents of the gland, the absolute activity of this enzyme per unit of weight became less from the fifth to the twentieth day of injection. An intense alkaline phosphatase reaction was observed by Dempsey, Greep, and Deane (1949) in the stroma and capillaries immediately underneath the epithelium of the seminal vesicle of the rat. After hypophysectomy the phosphatase activity declined and finally disappeared, but the rate of disappearance varied with different substrates. Similar results followed gonadectomy. Wislocki (1949) observed in the case of October deer alkaline phosphatase at the distal margins of the epithelial cells of seminal vesicle tissue, in the secretion filling the lumen, and in the interacinar stroma. In June deer much less enzyme activity was present.

The absence of this enzyme from the secretory epithelium of the seminal vesicles of the rat is in accordance with the findings of other investigators which indicate the absence or low concentration of alkaline phosphatase activity in the cytoplasm of cells responsible for globular protein secretion such as in salivary glands (Gomori, 1941), pancreas (Kabat and Furth, 1941), gastric mucosa (Jacoby, 1946), and albumen-secreting regions of the fowl oviduct (Bradfield, 1949). However, the occurrence of this enzyme in the interacinar stroma of this gland is in agreement with the results of earlier studies on collagen formation in wounds (Fell and Danielli, 1943) and bones (Bourne, 1942, 1943), rat uterine epithelium (Pritchard, 1947), the vaginal wall of ovariectomized mice treated with estrogen (Jenner, 1947), and silk glands of spiders and caterpillars (Bradfield, 1950). According to Bradfield (1950) the presence of alkaline phosphatase in these various tissues suggests a relationship between this enzyme and fibrillar protein formation. However, in a recent study Bern and Levy (1952) have presented data which cast doubt on the concept of a relationship between alkaline phosphatase activity and the synthesis of fibrous proteins such as keratin, collagen, and myosin.

In the intact animal the nucleus of the epithelium showed a strong reaction for acid phosphatase which became progressively weaker during the 20 days following castration as shown in Table 2. The cytoplasm and secretion granules likewise gave a strong reaction in the intact animal, but, as the cell atrophied following castration, the intensity of the reaction appeared to decrease in the cytoplasm and no secretion granules were present (Fig. 7). However, 5 days after hormone treatment the reaction was strong in the nucleus and cytoplasm and intense in the granules. The connective tissue and smooth muscle as well as the blood vessels in these tissues showed a decrease in acid phosphatase after castration followed by an increased reaction after hormone treatment (Table 2 and Fig. 8).

Porter and Melampy (1952) found that the acid phosphatase activity on a gland basis as determined by chemical analysis showed a 40% decrease 5 days following castration and a 65% reduction after 20 days. Likewise, during the same interval, alkaline phosphatase activity decreased 81%. There was a striking similarity between the decrease in activities of these enzymes and the loss in weight of the gland. The concentration of acid phosphatase increased 964% after 10 days of hormone injection.

Wislocki (1949) observed that acid phosphatase was localized almost entirely in the nuclei of epithelial cells and was found to a lesser degree in the nuclei of the stroma of the seminal vesicles of the deer. In the case of a June deer the acid phosphatase reaction was very much fainter than that found in October deer. Dounce (1950) has stated that acid phosphatase appeared to be generally present in both cytoplasm and nuclei. Wachstein (1944) in an investigation of renal phosphatases suggested that acid phosphatase was probably of importance in cell metabolism. This concept received further support when a marked increase in the enzyme was found in the perikaryon during chromatolysis (Bodian and Mellors, 1945), and Hard and Lassek (1946) have indicated that acid phosphatase may be of importance in maintaining the myelin sheath. The physiological significance of acid phosphatase in the tissues of the seminal vesicles of the rat is unknown but the activity of this enzyme was observed to be influenced either directly or indirectly by male-sex hormone.

SUMMARY

A basophilic reaction was observed in the cytoplasm of the secretory epithelium of the seminal vesicles of the rat following eosin-methylene blue and toluidine blue which was abolished by ribonuclease. The nucleoli were also basophilic but the reaction following ribonuclease was variable.

Alkaline phosphatase activity was absent from the nuclei, cytoplasm and secretory granules of the epithelium of the seminal vesicles of intact animals as well as castrate and hormone-treated castrates. The connective tissue, particularly the lamina propria and its blood vessels gave a strong reaction which became progressively weaker 10 days after castration.

Following 5 days hormone treatment the reaction became intense but after this time decreased. The results obtained from this histochemical study of both acid and alkaline phosphatase activity in seminal vesicle tissue agree with the chemical analysis of similar tissues for these enzymes. These enzymes were absent from seminal vesicle secretion. In the intact animal the nuclei of the secretory epithelium showed a strong reaction for acid phosphatase activity which became weaker following castration. Likewise the cytoplasm and secretion granules gave a strong reaction and the intensity appeared to decrease in the cytoplasm as the cells atrophied. However, after 5 days treatment with testosterone propionate (500 μg. per day) the reaction was strong in the nucleus and cytoplasm and intense in the granules. A similar response was also observed for acid phosphatase activity in the connective tissue, blood vessels, and smooth muscle of the gland.

The outer layer of the seminal vesicles of intact animals was composed of a fine, thin capsule of connective tissue; whereas the smooth muscle zone surrounding the lamina propria (interacinar stroma) was relatively thick and was interwoven with elastic tissue. The sites of localization of alkaline phosphatase closely paralleled the distribution of the lamina propria in this gland. Following castration there was an atrophy of the secretory epithelium and the connective tissue and muscle layers were thickened in appearance. The administration of testosterone propionate caused a reactivation of the gland.

Glycogen was absent from the seminal vesicle tissue of the rat. A slight positive reaction for periodic acid-reactive carbohydrates was obtained in the cytoplasm of some of the cells of the secretory epithelium, smooth muscle layer, the elastica interna of the blood vessels, and lamina propria.

It was found by the use of the colchicine technique in both intact animals and 20-day hormone treated castrates that 0.3% of the secretory cells showed mitoses. After one day of injection there was 0.2% dividing cells but following 2 days of hormone treatment there was 13.4%. Two days after gonadectomy a few of the nuclei of the secretory cells were pyknotic and contained small nucleoli; whereas 18 days later many were pyknotic and in numerous cases the nucleoli were absent or greatly reduced in size. After hormone treatment, both structures resembled those of intact animals.

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EFFECT OF IODIDE AND THYROID ON THE PRODUCTION OF TUMORS OF THE THYROID AND PITUITARY BY PROPYLTHIOURACIL[1]

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IN EXPERIMENTS carried out in this laboratory benign and metastasizing adenomatous tumors of the thyroid gland of rats have been found following prolonged administration (over one year) of propylthiouracil. Similar results have been reported previously by other investigators with thiourea (Purves and Griesbach, 1947; Money and Rawson, 1947), and with thiouracil (Dalton, Morris and Dubnik, 1948; Money and Rawson, 1950). The pathogenesis of the tumors is probably related to an increased production of thyrotropic hormone by the anterior pituitary over a prolonged period. The production or release of other hormones may also be affected, with resulting changes in other endocrine glands.

Ferguson and Sellers (1949) found that the concurrent administration of iodide with thiouracil for short periods (2–6 weeks) lessened the goitrogenic effect and resulted in thyroid glands which were 20–30% lighter than those produced by the goitrogen alone. They attributed this effect to the antithyrotropic action of iodide. This suggested that giving iodide with a goitrogen for a long period might affect the development of tumors produced by the goitrogen. Because of the antithyrotropic activity of the thyroid hormone itself, and in an attempt to maintain the metabolic and growth rates normal, in the experiments to be described dried thyroid was also given with propylthiouracil, the goitrogen selected for the investigation. The result of this treatment was an even greater enlargement of the thyroid gland, and in addition the occurrence of multiple adenomas of the anterior lobe of the pituitary.

EXPERIMENTAL

Two hundred and eighty rats of the Wistar strain, of equal sex distribution, bred in the laboratory, were divided into four groups and were given food and water *ad libitum*. Twenty from each group were kept in individual cages in order to follow the daily intake of food, and the remainder were kept in groups of ten in a cage. Each animal was weighed at weekly intervals.

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[2] Holder of a National Research Council of Canada Medical Fellowship, 1951–52.

Group 0 was fed the usual laboratory ration[3] in powdered form.

Group 1, laboratory ration plus propylthiouracil 0.02%.

Group 2, laboratory ration plus propylthiouracil 0.02% plus sodium iodide 0.02%.

Group 3, laboratory ration plus propylthiouracil 0.02% plus dried thyroid[4] 0.02%

The rats were maintained on this regime for 15 months after which time four rats from each group, chosen at random, were sacrificed at weekly intervals. At necropsy, body and organ weights were recorded and tissues were fixed in Bouin's solution or in formol-saline. After sectioning, tissues were stained with haematoxylin and eosin invariably, and in many cases with special stains for connective tissue, fat, ceroid, cytoplasmic granules, mast cells, etc. The pituitaries were stained with acid fuchsin, methyl blue (Wilson 1952) and often with Gomori's aldehyde-fuchsin stain. (Gomori, 1950). During the experiment the oxygen consumption of representative animals was measured at intervals using the method previously referred to by Sellers and You (1950). The level of iodine bound to plasma-protein was estimated (Barker, 1951) prior to sacrifice in representative groups, and the total iodine content of the thyroid (Kendall, 1920) was measured using that part of the gland not required for histological purposes.

Measurement of radio-iodine uptake (I^{131}) was carried out, and radioautographs were prepared following the method of Leblond and Gross (1948).

Measurement of exophthalmos

At necropsy, photographs were taken from vertically above the head of each rat after removal of the skin. The presence of exophthalmos was measured by dividing the distance between the points of maximum curvature on each cornea, by the distance between the malar processes. If the result was greater than 1, exophthalmos was said to be present; if less than 1, there was no exophthalmos. If the ratio was 1, or the angle of the head made measurements inaccurate, the reading was classed as "doubtful." The results are shown in table 4.

RESULTS

In analyzing the data it was found that expressing the results as a median and range presented more information in concise form than did the average and standard deviation. Occasional very large values rendered the latter method unwieldy, and difficult to interpret.

Rats which received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) gained very little weight during the entire experiment. The fur was coarse and sparse. The weight-curve of the group which received propylthiouracil plus thyroid extract (group 3) resembled that of the controls and at the end of the experiment the average weight was only slightly less than the control value (Table 1). The fur was normally sleek in appearance. In all groups the average food intake was consistent with the average gain in weight, the controls, and rats receiving propylthiouracil plus thyroid consuming almost twice the amount eaten by the other groups.

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[3] Fox breeder cubes, Master Feeds, Toronto Elevators Limited, containing 2.6 mg. iodine per 100 gm.

[4] Dried thyroid powder containing 0.3% iodine, Parke-Davis and Company, Walkerville, Ontario.

Thyroid

Weights: (Table 1). The addition of propylthiouracil to the diet caused a significant increase in the weight of the thyroid gland. When propylthiouracil and iodide were administered simultaneously the increase in weight was less than that observed with propylthiouracil alone, but when propylthiouracil and thyroid extract were administered simultaneously (group 3), the thyroid glands were tremendously enlarged to as much as eighty, and on the average to twenty times the weight of the thyroids of the control animals.

Pathology. Gross: The thyroid glands from animals of the three groups which had been fed propylthiouracil were all enlarged to a variable extent. In some cases the enlargement was diffuse, regular and involved both lobes. In others, one or both lobes had multiple small firm nodules or an occasional marble-sized cystic swelling (Fig. 1). The color was also variable, although glands from groups 1 and 3 (propylthiouracil and propylthiouracil plus thyroid) were deeper red and apparently more vascular than glands from group 2 (propylthiouracil plus iodide).

Microscopic: The microscopic appearance varied greatly within and between treated groups. Large areas (comprising most of the gland) showing

TABLE 1. WEIGHTS

No. of rats	Group	Sex	Body weight in grams			Thyroid weight, mg.		Pituitary weight, mg.		Adrenal weight, mg.	
			Initial av.	Final							
				Median	Range	Median	Range	Median	Range	Median	Range
15	0	M	152	399	340–484	36.0	27.4– 47.4	11.8	9.2– 18.0	37.9	27.2–45.8
14	control	F	144	275	254–324	28.5	20.2– 39.0	14.7	10.8– 35.7	55.6	28.0–78.8
20	1	M	159	191	142–278	97.7	44.6– 395.2	12.2	7.8– 17.0	24.0	18.1–32.1
17	PTU	F	145	175	119–230	79.8	40.2– 568.0	15.0	9.0– 22.4	24.2	18.2–36.6
14	2	M	166	189	148–211	50.1	27.6– 143.4	11.6	8.2– 15.6	24.2	19.2–29.0
12	PTU+I	F	152	158	108–212	48.6	32.0– 94.0	15.3	7.8–500.0	24.8	18.0–34.6
12	3	M	152	346	278–402	517.1	363.8– 674.1	20.9	10.0–164.0	39.9	32.4–54.0
12	PTU+Th	F	144	257	154–302	442.2	150.0–1660.0	28.6	11.6– 60.6	68.8	42.0–89.4

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EXPLANATION OF PLATE I

FIG. 1. Thyroid (wt. 1660 mg.) and pituitary (wt. 22.8 mg.) glands. Propylthiouracil plus thyroid group. Thyroid metastases found in lung. Note small nodule on pituitary (upper right, see figs. 11, 13).

FIG. 2. Pituitary gland (wt. 38 mg.) same group. Note nodule attached to anterior lobe (entire left of photograph). See figs. 16, 17.

FIG. 3. Thyroid. Small circumscribed area containing colloid. (Mallory×40.)

FIG. 4. Thyroid. Colloid "lake." Compressed follicular cells form a pseudo-capsule. (H.+E.×40.)

FIG. 5. Thyroid. Follicular cells surrounding well-stained colloid are stratified and hyperchromatic. (H.+E.×560.)

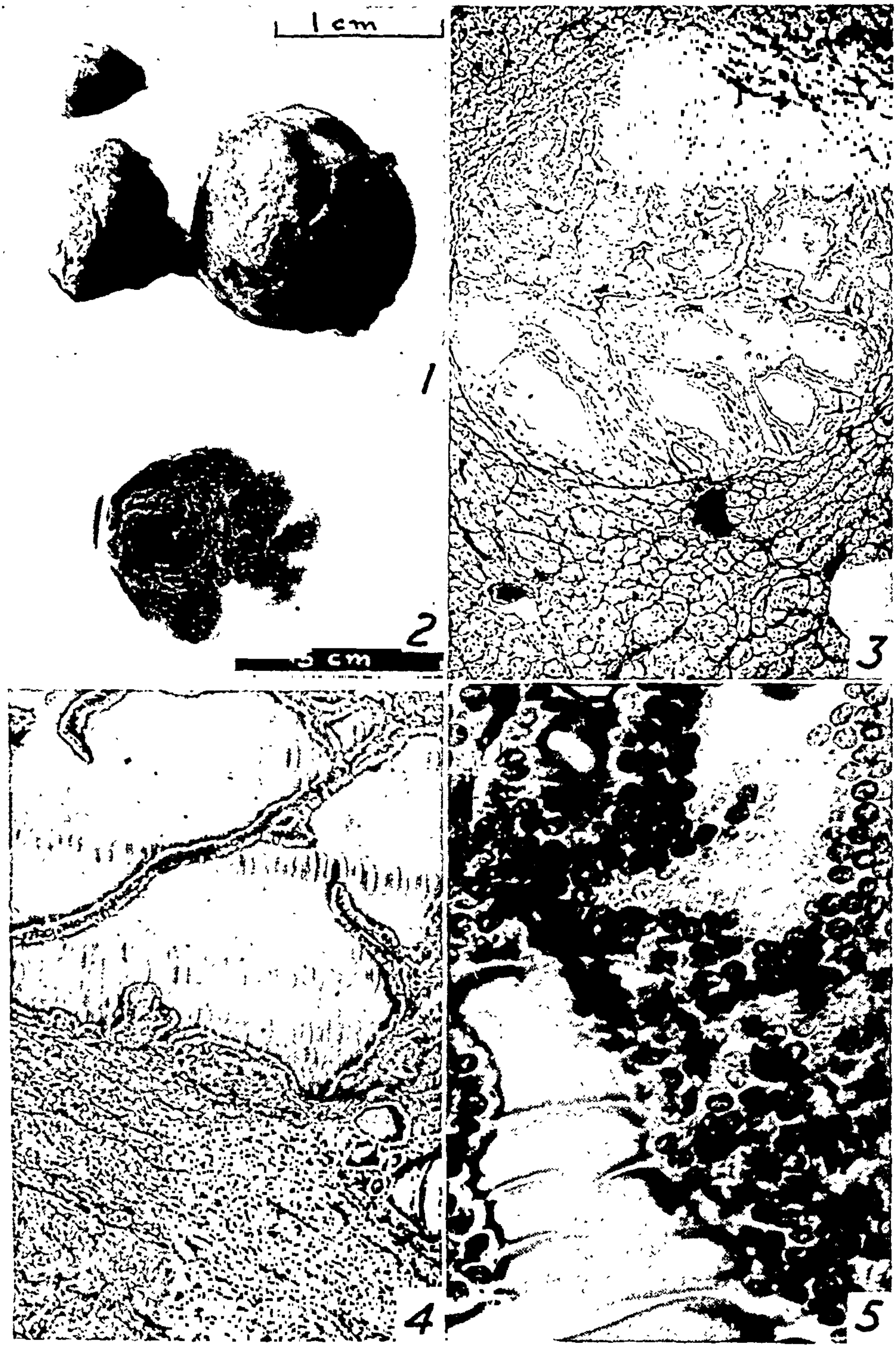

Plate I

hypertrophy and hyperplasia of the follicular cells, mitotic figures, budding of the follicular wall (with an apparent increase in number of follicles), vacuolation of, or more frequently almost complete disappearance of colloid could be found in every section. Generally these changes were advanced so that the normal architecture of the gland was no longer discernible.

Frequently (in almost every gland weighing more than double the average weight of the normal) an area made up of large follicles containing well stained colloid was demarcated from its surroundings by a pseudocapsule made up of compressed follicular cells or of fibrous trabeculae. The cells within such a nodule showed considerable variation in that sometimes they appeared cuboidal or even flattened, and in other sections, hyperchromatic, columnar cells in layers with many mitotic figures projected and branched into large colloid cysts or "lakes." Wide blood-sinuses, and evidence of hemorrhage into the cysts could be seen. The similarity of some areas to the papillary cystadenomas of human thyroids was a striking feature. Less frequently, circumscribed or semi-circumscribed areas, with little or no colloid and either vacuolated, but hyperchromatic cells, or anaplastic cells were evident (Figs. 3–7).

While the size of the glands (and of gross colloid cysts) was much greater in the group receiving propylthiouracil with thyroid the *presence* of localized colloid areas, of colloid cysts, or of cellular adenomas was not restricted to any one group receiving propylthiouracil. By histological examination it was not possible to assign any tissue-section chosen at random to its correct group.

In 3 or perhaps 4 animals (one—group 1, propylthiouracil; 2 or 3—group 3, propylthiouracil plus thyroid) metastases were found seeded through the lungs. Thyroid follicles containing colloid were found invading the capsule of the gland, in venules in muscle adjacent to the gland, in the

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Explanation of Plate II

Fig. 6. Thyroid. Projections from the follicular walls extend into dilated blood sinuses. Note the thin "stem" of the daughter follicle (center). (H.+E. ×150.)

Fig. 7. Thyroid. Hemorrhage has occurred into follicles. In some follicles (upper) necrosed follicular cells, blood pigments and colloid are present. Organization of smaller follicles has taken place. (H.+E. ×40.)

Fig. 8. A follicle of thyroid tissue in a venule adjacent to the gland. Note the intimal layer of the vessel, the red blood cells between it and the follicle, the colloid in the follicle. (Mallory ×900.)

Fig. 9. Thyroid tissue in the submucosa of the esophagus. The epithelium (lower left) and muscular layer (upper) can be seen on either side of the thyroid tissue. (Mallory ×150.)

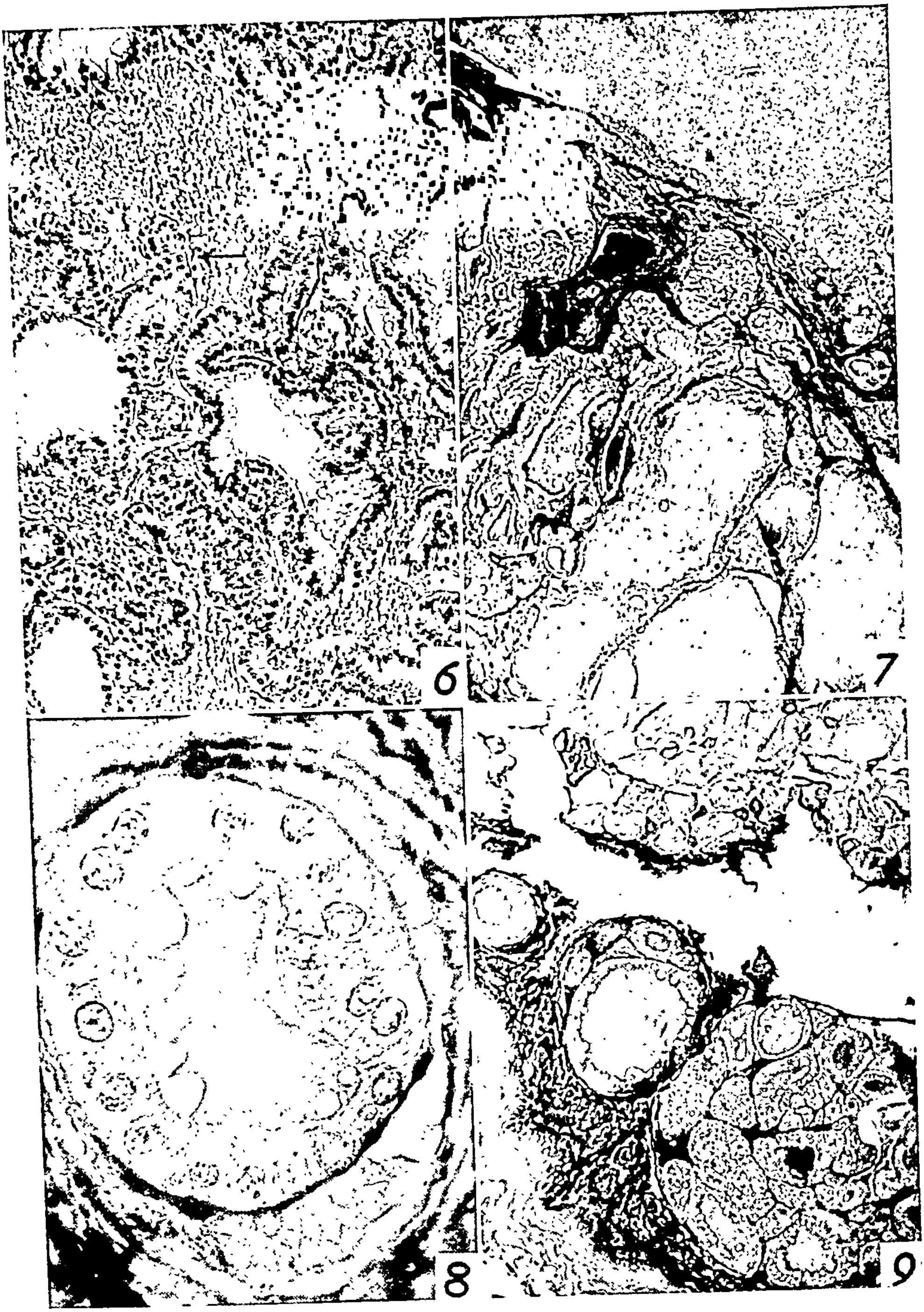

PLATE II

submucosa of the esophagus and in muscle deep to the esophagus, in a branch of the pulmonary artery in the lung and in numerous alveolar vessels (Figs. 8–10). In some instances the metastasizing thyroid tissue resembled normal gland while in other hyperchromatism, stratification, and frequent mitotic figures were seen.

Physiological and Biochemical Studies. Metabolic rates and protein-bound iodine: (Table 2)

The addition of propylthiouracil to the diet resulted in a fall in the metabolic rate and plasma protein-bound iodine values to hypothyroid levels. In group 2 (propylthiouracil plus iodide) the metabolic rate was even lower, but the protein-bound iodine was above the control value. The values obtained for rats which had received propylthiouracil plus dried thyroid (group 3) were slightly below those of the controls.

TABLE 2. METABOLIC RATE AND PLASMA PROTEIN-BOUND IODINE VALUES

Group	No. of rats	Metabolic rate		No. of rats	Protein-bound iodine	
		Average MR* (15 months)	% decreased from normal		Median, μg.	S.D.
0	16	150	0	13	4.63	±1.17
1	8	99	34	15	1.93	±0.61
2	7	65	57	12	6.93	±1.53
3	8	138	8	10	2.55	±0.62

* Cc. O_2/min./m^2 at N.T.P.

Radio-active iodine uptake:

The uptake of radio-active iodine (24 hours) calculated on a basis of concentration per unit of thyroid tissue was diminished in the rats which had been fed propylthiouracil (group 1). The uptake was less in the rats which had been fed propylthiouracil plus thyroid extract (group 3) and least in those rats which had received propylthiouracil plus iodide (group 2).

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EXPLANATION OF PLATE III

FIG. 10. Lung. Thyroid tissue in a branch of the pulmonary artery. The muscular wall of the vessel is seen in the lower left, and bronchial epithelium adjacent to the artery, in the upper right. (Mallory ×150.)

FIG. 11. Pituitary (see Fig. 1). Localized areas of chromophobic cells found in many rats of the propylthiouracil-thyroid group. (Acid fuchsin, methyl blue ×40.)

FIG. 12. Pituitary. Edge of a chromophobic area (right). The cytoplasm of the pseudocapsular cells is compressed and stains indefinitely, the nuclei are pyknotic. (Acid fuchsin, methyl blue ×500.)

FIG. 13. Pituitary. A higher magnification of chromophobic area shown in Fig. 11. Both cytoplasm and nuclei stain poorly, vary in size and shape. In the photograph red blood cells appear black, one basophil can be seen (upper right). (Acid fuchsin, methyl blue ×500.)

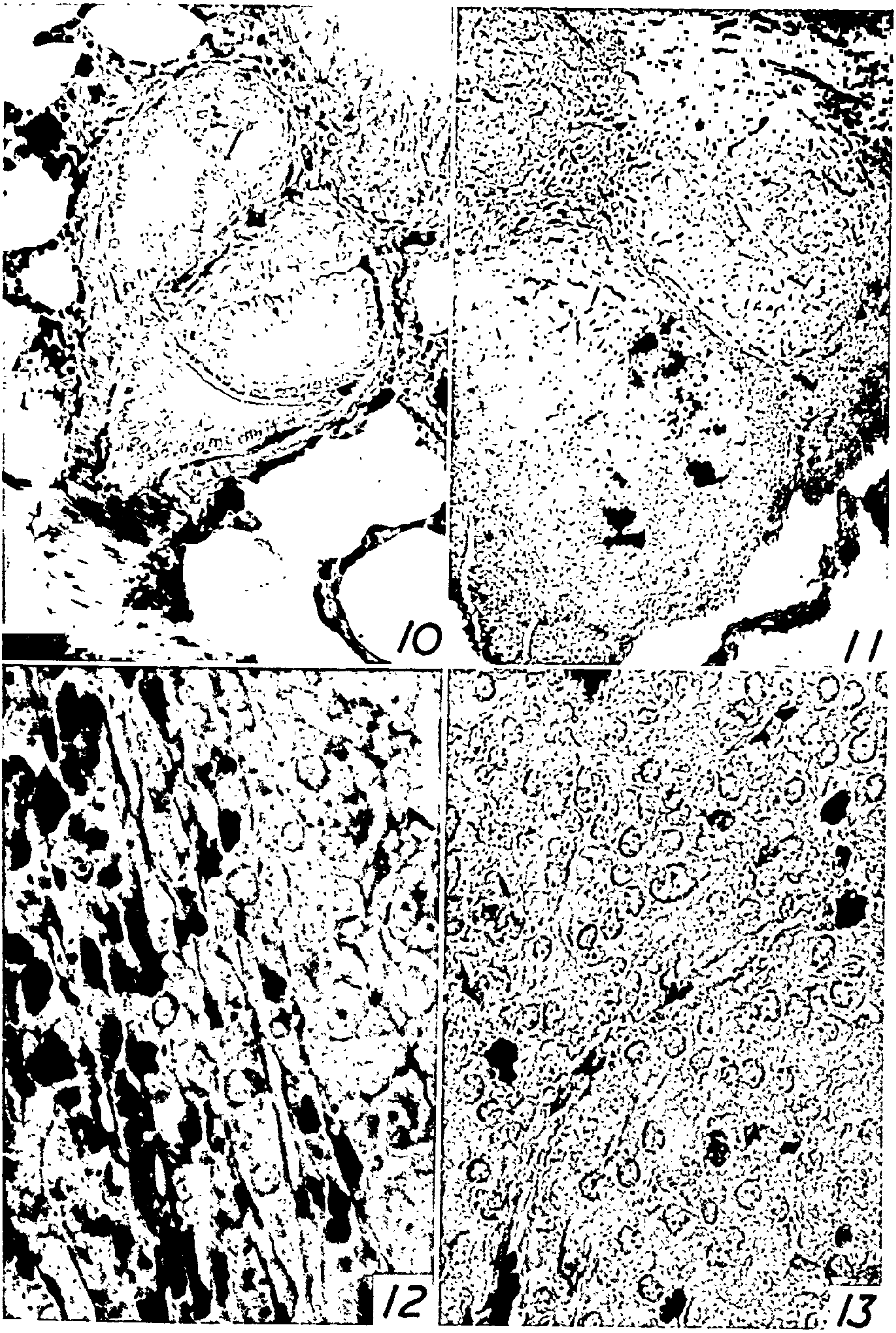

Plate III

Although only six animals in each group were studied, the differences were significant. No significant difference existed when the calculation was carried out for total weight of thyroid tissue.

Radioautographs (24 hours) showed little or no concentration in the three groups which received propylthiouracil. The difference between groups, if any, could not be quantitated satisfactorily. There was no apparent concentration of iodine in the deeply stained colloid of the adenomas.

Iodine content of the thyroid gland: (Table 3)

The iodine content of the thyroid gland was very low in the rats which had been fed propylthiouracil (group 1). The level of iodine was higher in those rats that had received propylthiouracil plus iodide (group 2). In the group which had been given propylthiouracil plus thyroid extract the iodine content of the whole thyroid was found to be only one-third less than that of the controls, although the value was very low when calculated per unit weight of thyroid tissue.

TABLE 3. IODINE CONTENT OF THYROID

Group	No. of rats	Iodine content (μg. per 100 mg. wet tissue)		Iodine content (μg. iodine per gland)	
		Median	Range	Median	Range
0	21	130.4	88.4 –196.40	46.6	18.2 –58. 3
1	29	2.41	0.41– 11.40	2.74	1.08– 6.86
2	18	23.68	8.32– 39.89	11.83	7.20–20.40
3	13	5.64	2.60– 10.19	27.68	21.50–43.20

Pituitary

Weights: (Table 1). The pituitaries of the rats which had received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) were no heavier than those of the control animals. The addition of thyroid extract to the propylthiouracil (group 3) caused a significant increase in the weight of the pituitaries.

Pathology. Two of the pituitary glands from rats which received propylthiouracil plus thyroid extract (and one, the largest of all, from a rat receiving propylthiouracil plus iodide) were large (pea-sized), friable and dark red in color. Most of the glands from this group (Figs. 1 and 2), although somewhat larger than usual, looked normal except that in many instances there were one or more small flattened reddish nodules on the infero-lateral surfaces of the anterior lobe. The larger of these were as big as a normal pituitary (rat).

Microscopic: There was a consistent degranulation of the eosinophil cells in the treated groups which varied in degree from animal to animal. In spite of this it was usually, but not invariably, possible to find some cells which contained eosinophilic granules in part of a section. Basophils were almost always degranulated; and often the cells were enlarged and the cytoplasm was vacuolated. In most sections from animals of treated groups a considerable number of very large cells showing extreme vacuolation of the cytoplasm but no granulation could be seen. The staining characteristics of the cytoplasm varied but usually stained pale blue with basic stains.

There was a relative increase in chromophobic cells.

In a high proportion of the propylthiouracil plus thyroid group, areas which stained very poorly were demarcated from normal tissue by a pseudocapsule of compressed cells (Figs. 11, 12). These areas were made up of masses of "chromophobic" cells, the nuclei of which varied in size and appearance from small, deeply stained, almost pyknotic, to very large hypochromatic structures. In a few sections faint eosinophilic granulation could be made out in the cytoplasm. The cytoplasm was pale, profuse and the cell borders were irregular (Figs. 13, 16, 17). The cytogenesis of the cells is not clear, and in spite of the chromophobic nature of the cells we are inclined to believe that they may have originated from chromophil cells. The evidence for this is not entirely convincing and consists of finding eosinophilic and less often basophilic transitional forms (Figs. 16, 17).

In the large hemorrhagic tumors found grossly, the microscopic appearance showed chromophobic cells interspersed with numerous mast cells (Figs. 14, 15). No "transitional" forms were seen in these instances. Areas of hemorrhage and necrosis were present and these contained deposits of "ceroid" demonstrable by special staining.

Adrenals

Weights: (Table 1). The adrenals of the female rats (but not the male) which had received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) weighed less than those of the control animals. Those rats which had received propylthiouracil plus thyroid extract had adrenal glands which weighed approximately the same as those of the control animals.

Pathology. The adrenal glands of most of the rats in the groups treated with propylthiouracil, or propylthiouracil plus iodide showed involutionary changes in the zona reticularis (Fig. 18). The addition of dried thyroid to the diet (group 3) prevented this involutionary change.

Exophthalmos

In Table 4 it is seen that a high percentage of animals receiving propylthiouracil became exophthalmic. Dried thyroid reduced the incidence very greatly but iodide was ineffective.

DISCUSSION

Rats which received propylthiouracil plus thyroid extract achieved an almost normal growth curve. This suggests that the depression of growth observed in the rats to which propylthiouracil or propylthiouracil plus iodide was given was due to a lack of thyroid hormone (Hammett, 1926) rather than to a direct inhibitory effect of proplythiouracil on the growth hormone of the anterior pituitary (Williams *et al.*, 1944).

The addition of a relatively large amount of iodide (approximately 1.5 mg. sodium iodide per rat per day) to the diet containing propylthiouracil reduced the goitrogenic effect of propylthiouracil, although the reduction of metabolic rate was even greater than in the group which received propylthiouracil alone.

TABLE 4. EXOPHTHALMOS

Group	No.	Exophthalmos			Incidence (per cent)
		Present	Absent	Doubtful	
0 (control)	25	0	24	1	0
1 (PTU)	35	29	4	2	83
2 (PTU+I)	22	17	4	1	77
3 (PTU+Th)	16	3	12	1	19

Thyroid

An unexpected finding was the tremendous enlargement of the thyroid gland when dried thyroid (approximately 3 mg. per day) was added to the diet containing propylthiouracil. This amount of thyroid had been chosen in an attempt to maintain the growth rate and metabolic rate within nor-

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EXPLANATION OF PLATE IV

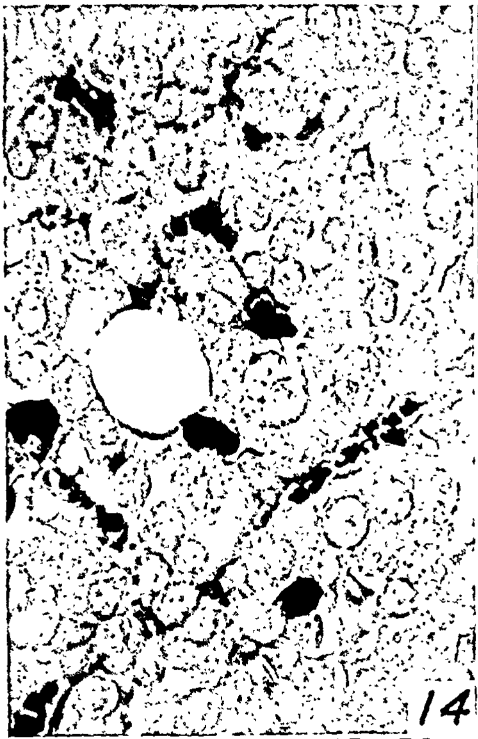
FIG. 14. Pituitary, Chromophobic adenoma. Variation in nuclear and cell size is apparent. Nuclear disintegration, extreme vacuolation of the cytoplasm and in other sections hemorrhage and necrosis are seen. The cells which appear black in photograph are mast cells. (Acid fuchsin, methyl blue×500.)

FIG. 15. Pituitary. Chromophobic adenoma. Large purple granules (black in photograph) cover the nuclei of cells having all the staining characteristics and appearance of mast cells. (Basic fuchsin—fast green×1000.)

FIG. 16. Pituitary (gross fig. 2). Chromophobic area with large vacuolated cells, showing no distinctive granulation, stained pale blue with basic stains. (Acid fuchsin, methyl blue×1280.)

FIG. 17. Another field of same pituitary showing transitional chromophil cells. Upper cells (B) are basophils; lower cells (A) are eosinophilic. (Acid fuchsin, methyl blue×1280.)

FIG. 18. Adrenal. At lower right the large pale chromaffin cells are normal medullary tissue. Approximating this area atrophied cells of the reticularis, surrounded by black reticular fibres can be seen. (Mallory×500.)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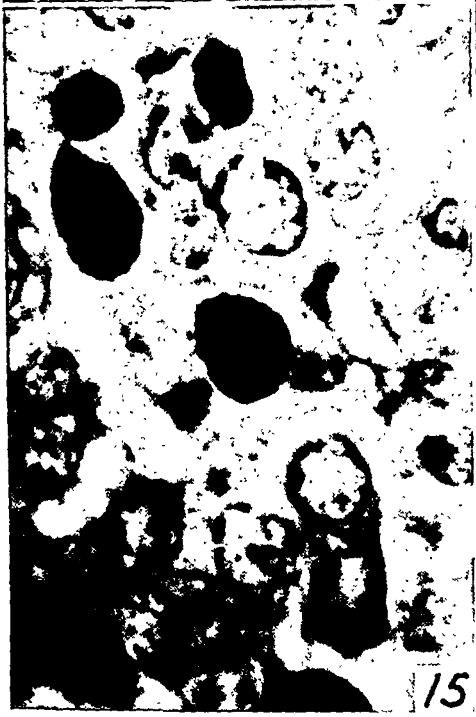
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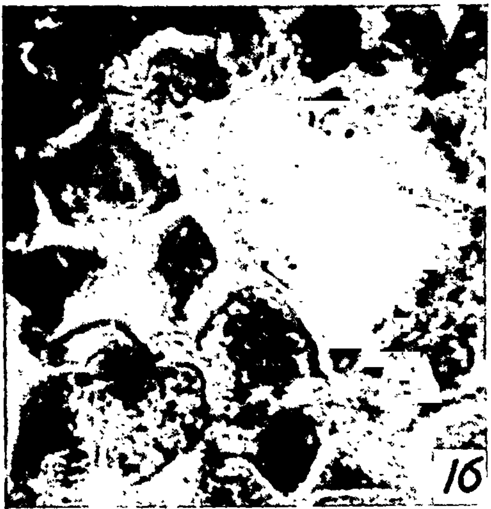
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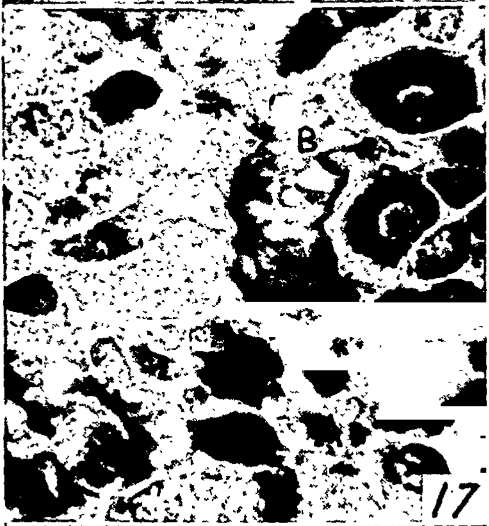

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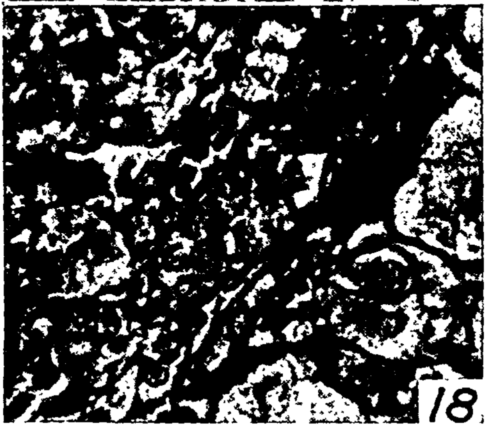
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Plate IV

mal limits in spite of the continued administration of propylthiouracil. Both of these values were slightly lower than those of the control group but fell within the range of "normal." Nevertheless, the size of the thyroid, and of the pituitary was significantly greater than in the other groups. It is necessary to postulate that there was some substance in the thyroid extract which stimulated the pituitary and the thyroid gland, or that the thyroid hormone supplied as dried thyroid in the diet was less than necessary to inhibit the pituitary, and a "hormonal imbalance" existed. Other evidence at hand, showing that in lengthy experiments of this nature, relatively large amounts of crystalline l-thyroxine added to the diet are necessary to prevent enlargement of the thyroid, suggests that the second alternative is more likely. It is possible that the thyroid hormone exerts a stimulatory function (under some circumstances) on the cells of the pituitary and thyroid gland. Unfortunately we have no information on the thyrotropin content of the anterior pituitary under the conditions described here. The great enlargement of the thyroid, and the production of metastasizing adenomas, suggest that continued stimulation of the thyroid gland by the thyrotropic hormone is the important causal factor (Purves and Griesbach, 1949; Money and Rawson, 1950).

The spread of the tumors appears to be through vascular channels. Apart from local invasion of the capsule of the gland, metastases were found exclusively as intact thyroid follicles in blood vessels in the thyroid, in venules adjacent to the thyroid, in branches of the pulmonary artery, and in alveolar vessels. No attempt was made to transplant or culture the tumors, but from other somewhat similar studies (Morris, Dalton and Green, 1951; Hall and Bielschowsky, 1949; Bielschowsky *et al.*, 1949) it would appear that they are at least *potentially* malignant.

Pituitary

The development of chromophobe adenomas of the pituitary in mice following destruction of the thyroid by radio-iodine has been reported by Furth (1952). As far as we know, no investigators have observed such tumors subsequent to the administration of goitrogenic drugs. Beyond compression of the base of the brain, not even the largest showed evidence of infiltration of adjacent tissue, or metastatic spread. The tumors were made up of chromophobic cells of very variable size interspersed with mast cells. Areas of hemorrhage and necrotic tissue (containing ceroid) were present in the larger ones. It has already been observed that in our opinion this staining characteristic does not necessarily mean that they arose from chromophobe cells. Indeed our present view tends to oppose this.

The metabolic rate and protein-bound iodine were lower in the group receiving propylthiouracil than in the controls. The lower metabolic rate obtained when iodide was given in addition to propylthiouracil is difficult to explain but is perhaps related to the relatively high dose level of iodide. The higher value obtained for the protein-bound iodine in this group probably reflects the higher intake of iodide and has been observed by others (Barker, 1948). In the group receiving thyroid the protein-bound iodine value was lower than normal, which supports the hypothesis that the amount of thyroid given was inadequate to replace that normally supplied by the gland.

Iodine content of the thyroid

The low iodine content of the thyroids from animals which received propylthiouracil, and the absence of concentration of iodine shown by radioautography was not unexpected. The differences between groups (in iodine content) are appreciable but cannot be explained satisfactorily from available information. The finding of granular eosinophil cells in the pituitaries of *all* the treated groups would imply (Purves and Griesbach, 1947) that thyroidal or extrathyroidal production of some thyroid hormone was taking place in both the propylthiouracil and propylthiouracil plus iodide groups. If such were the case, differences in iodine take-up and content might be expected with the differing treatments.

Exophthalmos

The production of exopthalmos in rats by feeding propylthiouracil has been reported previously (Sellers and Ferguson, 1949). This finding which occurred in a high proportion of the animals treated with propylthiouracil, or propylthiouracil plus iodide, was much less common in those animals which were given dried thyroid. Without knowing the amounts of thyrotropin produced or released it is not possible to comment on its possible role in the causation of exophthalmos. From the size of the thyroid glands, one would assume that the amount of thyrotropin available was large. Nevertheless, administered thyroid effectively prevented the exophthalmos.

SUMMARY

For a period of 15 months or more Wistar rats were fed the usual laboratory ration supplemented with (1) propylthiouracil, 0.02%, (2) propylthiouracil plus 0.02% sodium iodide, (3) propylthiouracil plus 0.02% dried thyroid.

Growth and metabolic rates were much lower than normal with propylthiouracil or propylthiouracil plus iodide but were nearly normal with propylthiouracil plus thyroid.

The thyroid glands were enlarged greatly in the three treated groups; the differences in gland size between each group (including the control)

were significant. The thyroids of rats which received iodide showed the least enlargement and those of rats which received dried thyroid with propylthiouracil the greatest (up to 80 times normal).

Histologically, in addition to the hyperplasia and disappearance of colloid typical of propylthiouracil administration, circumscribed areas of large follicles containing colloid were present. The cellular detail and apparent "activity" in these adenomas varied greatly. The particular treatment did not appear to control the type of adenoma produced. The radioiodine uptake was low in treated groups. There was no apparent concentration of iodine in the deeply stained colloid of the adenomas (24 hour radioautographs).

In 3 or perhaps 4 cases metastatic thyroid tissue was found in the lungs. Spread was by vascular channels as metastases were found in venules of thyroid, esophagus, muscle, and in a branch of the pulmonary artery. No attempt at transplantation or culture was made.

The pituitaries of rats receiving propylthiouracil plus thyroid were significantly heavier than those of other groups. Single or multiple "chromophobic" adenomas occurred frequently in this group.

The eosinophils of the pituitaries of all treated groups were incompletely degranulated. Generally the basophils were degranulated, large and vacuolated.

It is suggested that the tremendous increase in size of the thyroid gland, the great enlargement and adenomas of the pituitary, in the propylthiouracil plus thyroid group may be ascribed to a hormonal imbalance rather than to some unknown substance in the dried thyroid. The thyroid hormone itself may exert some stimulating function on the cells of the pituitary and thyroid.

Exophthalmos occurred in most (about 80%) of the rats receiving propylthiouracil or propylthiouracil plus iodide. It occurred in about 20% of those receiving propylthiouracil plus thyroid.

The adrenal glands of female rats receiving propylthiouracil, propylthiouracil plus iodide, but not propylthiouracil plus thyroid, were significantly lighter than those of the controls. Histologically the reticularis showed involutionary changes.

Acknowledgment

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BIOASSAY OF RELAXIN

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IN 1926 Hisaw first demonstrated experimentally the relaxation of the pelvic ligaments of guinea pigs during estrus following the injection of blood serum from pregnant rabbits. Later work indicated a specific hormone, designated as relaxin, was responsible for the looseness of the symphysis pubis, since the effect was also observed following injection of extracts of sow corpora lutea into castrated guinea pigs provided the animals were pretreated with estrogen. Since that time, most of the experimental work on relaxin has been based upon bioassays with this all-or-none pelvic looseness endpoint determined by manual palpation. During this assay procedure, the possibility of traumatizing the loosened tissue of the pubic symphysis or exaggerating the hormone-induced separation cannot be excluded. The radiographic measurement of the inter-pubic gap would seem desirable because not only would this provide an objective record but the determination of the actual interpubic distance might provide a more sensitive assay. Hall (1947) and Talmage and Hurst (1950) have employed dental x-ray machines to demonstrate relaxin activity but few details of the actual technics employed have been published. Talmage and Hurst attempted to refine the conventional guinea pig assay by substituting radiographic measurements for manual manipulation. However, the variability of response of individual animals was so great that these investigators concluded the assay would be impractical. The use of x-rays to determine separation of the pubic symphysis seems justified because Hall (1947) has shown that in untreated pregnant mice the time of beginning of pubic separation coincides within a day or two with the first histological evidence of relaxin effects on the pubic tissues.

This paper represents an attempt to develop a more reliable bioassay for relaxin employing mice with radiographic evaluation of the response.

EXPERIMENTAL

Swiss albino virgin mice[1] were employed after ovariectomy, except in later trials when intact immature females were employed. After a five-

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[1] Albino Farms.

day postoperative period, each animal received daily intraperitoneal injections of 1.0 μg. estradiol benzoate in corn oil, usually for a total of eight days. This procedure was varied in some experiments as will be indicated. During the experiments where relaxin was injected over a period of more than one day, the estrogen injections were continued on a daily basis.

In order to standardize the positioning of the animal for radiography as well as to remove the operator from the area of harmful radiation, the holder, illustrated in Figure 1, was constructed.[2] In use, the lower abdomen of the mouse rests directly upon the film. The cephalic edge of the film is tilted upwards into the belly of the animal to approximate a right angle with the x-ray beam. The film left projecting from the rear of the holder conveniently is used for placing the lead numerals to identify the individual negatives. For the accurate radiographing of the inter-pubic gap, the x-ray beam must strike the symphysis sagitally and, in order to avoid the vertebral and tail bones, from some angle below the vertical. After the animal's hindquarters are fixed in the holder with the hinged restrainer bar, the tail is pulled upward and forward and wedged into the forked holder. This motion tilts the entire pelvis and tends to closely approximate the symphysis against the film. The focal spot of the x-ray tube was at least 35 cm. from the animal so that the image on the film could be assumed to represent the actual bony separation. Measurements obtained under these conditions agree closely with figures published by Hall (1948).

It was found that the consistent production of sharp radiographs which permitted accurate measurements was difficult. The x-rays pass through approximately 10–12 cm. of soft tissue and careful exposures were necessary to produce sufficient contrast with the thin bony parts for accurate visualization of the pubic gap. Since the sole aim of the x-ray technic was the production of an objective record of the separation of the pubic bones, the use of high kilovoltages, customarily employed in routine medical radiographic work to bring out detail, resulted in films with such gradual density transitions across the pubic symphysis that the precise placing of the caliper points was impossible. The quality of radiographic image required was the short-scale, high contrast type. The x-ray machine available for this work was a General Electric Portable, Model F, with a 10 milliampere capacity and a constant output of 63 kilovolts. By interposing a injecting the animals twice per day it was found that an 8–16 hour schedule was equivalent to a 12-12 hour schedule, and when injections were continued for periods up to nine days the maximum separation occurred at 2–5 days with increasing variable receptivity to relaxin. Therefore an assay period of 48 hours was selected as representing the time of optimal response. In an additional experiment the pubic response to four doses of relaxin administered at 12-hour intervals was compared to that of six doses given at 8-hour intervals. The data are presented in Figure 2. The total amount injected in each group was 1.0 G.P.U. per day and no significant difference was apparent. Therefore an 8-16-8-16 hour, twice per day for 48 hours, injection schedule for all assays was adopted.

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[2] The holder was made from 0.250″ sheet of Plexiglass with the hinged restrainer bar bent to shape by the cautious application of heat. An ordinary snap fastener of the type usually employed on canvas awnings was used to keep this bar fastened down when in use. The film slot was built up of wedge-shaped pieces to position the dental films approximately 20° from the horizontal. Two thin rabbeted pieces were cemented to the edges of the inclined wedge to accommodate dental films inserted with the long diameter parallel wth the holder.

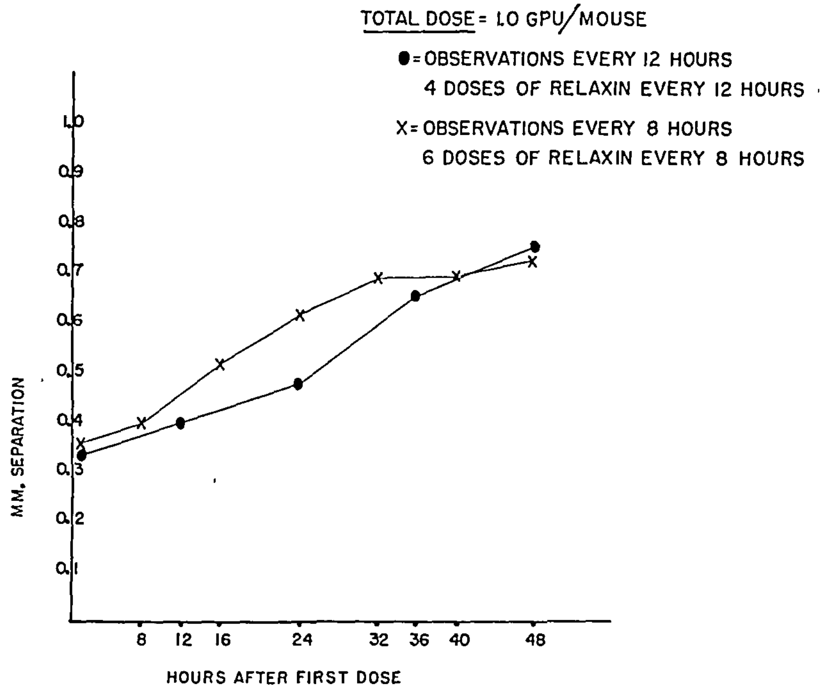

FIG. 2.

In a few experiments, treating the mice with ACTH, cortisone, progesterone, or desoxycorticosterone in addition to estrogen and relaxin resulted in no increment in response. Control groups pre-treated with estrogen and injected with saline showed no response.

Before each assay, a control radiograph was taken immediately prior to the relaxin injection. The average pubic separation of both ovariectomized and intact, immature female mice, as determined after estrogen pretreatment but prior to relaxin administration, ranged from 0.32 to 0.35 mm. About 15% of the ovariectomized animals had an initial pubic separation of over 0.5 mm., and although experience showed that these particular animals responded with an equal or greater relative separation than the remaining animals, they were arbitrarily discarded in order to reduce assay variation.

The pubic responsiveness to relaxin was not influenced by the age at which ovariectomy was performed between 30 and 73 days of age. No significant difference in response could be detected between the following varying conditions: Administration of estrogens subcutaneously and of relaxin intraperitoneally, both hormones administered subcutaneously, and finally the estrogen administered intraperitoneally and the relaxin subcutaneously.

TABLE 1. INFLUENCE OF NUMBER OF DAYS OF ESTROGEN PRETREATMENT ON PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:
Relaxin injections—2 per day for 2 days.
Observations—Pre-relaxin, 24 hours and 48 hours post-relaxin.
Estrogen—1 μg./day estradiol benzoate in corn oil.
Relaxin—0.5 G.P.U./day.

No. of estrogen inj. before relaxin treatment	No. of mice	Mean weight gm. ±S.E.	Pelvic separation		
			Pre-relaxin mm.	Post-relaxin 24-hrs. mm.	Post-relaxin 48-hrs. mm.
4	16	28 ±0.5	0.33 ±0.01	0.50 ±0.66	0.74 ±0.07
5	30	28 ±0.5	0.32 ±0.01	0.53 ±0.05	0.74 ±0.06
6	7	30 ±0.9	0.34 ±0.02	0.56 ±0.11	0.76 ±0.21
6	16	29 ±0.7	0.32 ±0.01	0.48 ±0.07	0.65 ±0.10
6	15	26 ±0.4	0.36 ±0.02	0.49 ±0.04	0.65 ±0.08
6	16	28 ±0.7	0.35 ±0.01	0.50 ±0.04	0.68 ±0.08
7	9	28 ±1.0	0.32 ±0.01	0.45 ±0.03	0.59 ±0.09
8	15	27 ±0.8	0.33 ±0.02	0.46 ±0.05	0.67 ±0.10

Estrogen pretreatment was varied from four to eight days as illustrated in Table 1. No significant difference in response could be observed.

An attempt was made to use the male mouse for the assay. As Table 2 illustrates, no significant pelvic response could be elicited even when doses as high as 1.2 G.P.U. per day were administered.

Attempts to establish dose-response curves are recorded in Tables 3 to 6. In Table 3 the response of increasing doses of relaxin from 0.15 G.P.U. to 0.60 G.P.U. per day was studied with an estrogen pretreatment period of six days. As little as 0.15 G.P.U. total dose produced a significant response in 24 hours, but when the dose within the day period, was increased to 0.30 or 0.60 G.P.U. only small increments in response were apparent. When the assay was run for two days, a relatively large difference between 0.15 G.P.U. per day and 0.30 G.P.U. per day was found. Increasing the dose to 0.60 G.P.U. per day in the two-day assay did not result in a significant increase in response. The data were also used in a

2×2 design according to Bliss (1944). Nine animals were used at each concentration of 0.15 and 0.30 G.P.U. per day. A potency ratio of 126.6% was found. The error at $P=0.95$ was +252% and −63%. The t value was 0.640 and the index of λ was equal to 0.426.

TABLE 2. MALE MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:

Days	1	2	3	4	5	6	7	8	9
Treatment	E*	E	E	E	E	E	E R**	E R	
Observation							O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Day 7 pre-relaxin mm.	Day 8 post-relaxin 24 hrs. mm.	Day 9 post-relaxin 48 hrs. mm.
0.15	13	28 ±0.7	0.32 ±0.01	0.32 ±0.01	0.32 ±0.01
0.30	15	26 ±0.6	0.32 ±0.01	0.33 ±0.01	0.34 ±0.01
0.60	16	28 ±0.6	0.32 ±0.01	0.36 ±0.02	0.46 ±0.05
1.20	20	29 ±0.7	0.30 ±0.01	0.34 ±0.01	0.34 ±0.01

*E = Estradiol benzoate in corn oil 1 μg./day.
**R = Chilcott Releasin 31-81D SF.

TABLE 3. MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:

Days	1	2	3	4	5	6	7	8	9
Treatment	E*	E	E	E	E	E	E R** R	E R R	
Observation							O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Day 7 pre-relaxin mm.	Day 8 post-relaxin 24 hrs. mm.	Day 9 post-relaxin 4-hrs. mm.
0.15	19	28 ±0.5	0.33 ±0.01	0.41 ±0.03	0.57 ±0.04
0.30	30	28 ±0.6	0.33 ±0.01	0.53 ±0.05	0.91 ±0.10
0.60	13	31 ±2.4	0.33 ±0.02	0.55 ±0.10	0.93 ±0.12

* E = Estradiol benzoate in corn oil 1 μg./day.
** R = Chilcott Releasin 31-81D SF.
Data of Day Nine.
$X = 100$ dose per day.
$Y = 1.16\ (X - 1.36) - 0.78$.
Doses from 0.15 to 0.30 G.P.U./day.

Additional dose-response data are presented in Tables 4, 5, and 6. In Tables 4 and 5, immature intact female mice were used in place of ovariectomized animals. When two doses of estrogen were employed daily (Table 5) a reasonably linear response was observed over the range 0.05 to 0.50 G.P.U. per day. With one dose of estrogen per day, the useful range was 0.05 to 0.25 G.P.U. per day.

Table 6 summarizes the dose-response data using ovariectomized females with an estrogen pretreatment period of five days. The slope of 0.60

is favorable and the useful range appears to be between 0.10 to 0.80 G.P.U. per day.

The utility of the method (see Table 6) was further tested by the simplified 2×2 design of Bliss. Two concentrations of each of the unknown and standard were 0.25 and 0.50 G.P.U. per day. Since the same material was used for both the unknown and the standard, the theoretical potency ratio was 100. When ten animals were used at each concentration for both unknown and standard a potency ratio of 77% was found. The t value for

TABLE 4. RELAXIN IN IMMATURE, INTACT FEMALES (28 DAYS AT START)

Injection—observation schedule:

Days	1	2	3	4	5	6
Treatment	E*	E	E	E R** R	E R R	E
Observation				O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Pelvic separation Day 4 pre-relaxin mm.	Pelvic separation Day 5 post-relaxin mm.	Day 6 post-relaxin mm.
0	8	16 ±0.9	0.34 ±0.01	0.34 ±0.01	0.34 ±0.02
0.05	13	18 ±0.5	0.35 ±0.01	0.40 ±0.02	0.38 ±0.03
0.10	14	17 ±0.4	0.32 ±0.01	0.48 ±0.04	0.51 ±0.06
0.25	17	16 ±0.7	0.36 ±0.01	0.53 ±0.05	0.61 ±0.05
0.50	18	16 ±0.6	0.33 ±0.01	0.47 ±0.03	0.56 ±0.05

* E = Estradiol aqueous.
** R = Chilcott Releasin 31-81D SF.
Data of Day Six
$X=100$ (dose per day).
$Y=0.57\ (X=1.07)+0.51.$
Doses from 0.05 to 0.25 G.P.U./day.

TABLE 5. MOUSE PELVIC RESPONSE TO RELAXIN IN IMMATURE, INTACT, FEMALES (28 DAYS AT START)

Injection—observation schedule:

Day	1	2	3	4	5	6
Treatment	E* E	E E	E E	E+R** E+R	E+R E+R	
Observation				O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm ±S.E.	Day 4 pre-relaxin mm.	Day 5 post-relaxin mm.	Day 6 post-relaxin mm.
0.05	21	17 ±0.4	0.32 ±0.01	0.34 ±0.01	0.37 ±0.01
0.10	18	17 ±0.8	0.32 ±0.01	0.40 ±0.03	0.50 ±0.06
0.25	20	17 ±0.04	0.33 ±0.01	0.53 ±0.05	0.71 ±0.07
0.50	40	17 ±0.04	0.35 ±0.01	0.69 ±0.06	0.84 ±0.08

* E = Estradiol aqueous.
** R = Chilcott Releasin 31-81D AF.
Data 6
$X=100$ (dose per day).
$Y=0.412\ (X-1.30)+0.65.$
Doses from 0.05 to 0.50 G.P.U./day.

significance of difference of slopes was 0.915 showing that the slopes were not significantly different, and at P = 0.95, the expected error determining the potency ratio was −49% and +96%. When the number of animals was increased to 16 in each group, the potency ratio was found to be 91.5% and the error at P = 0.95 was −35 to +55%. The index of precision λ was equal to 0.375. The t test for differences between slopes was 0.673 which means that the slopes of unknown and standard were not significantly different.

TABLE 6. MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule

Days	1	2	3	4	5	6	7	8
Treatment	E*	E	E	E	E	E R** R	E R R	
Observation						O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm ±S.E.	Pelvic separation		
			Day 6 prerelaxin mm.	Day 7 post-relaxin 24 hr. mm.	Day 8 post-relaxin 48 hr. mm.
0	21	29 ±0.6	0.30 ±0.01	0.33 ±0.03	0.29 ±0.01
0.10	17	27 ±0.5	0.30 ±0.01	0.33 ±0.03	0.37 ±0.05
0.25	19	26 ±0.6	0.31 ±0.01	0.42 ±0.04	0.50 ±0.05
0.50	30	28 ±0.5	0.32 ±0.01	0.53 ±0.05	0.74 ±0.06
0.60	21	30 ±0.6	0.33 ±0.01	0.49 ±0.06	0.77 ±0.09
0.80	12	31 ±0.5	0.30 ±0.01	0.76 ±0.14	1.03 ±0.13
1.00	7	28 ±0.9	0.37 ±0.02	0.66 ±0.10	1.10 ±0.20
4.00	9	28 ±0.6	0.33 ±0.02	0.87 ±0.18	1.20 ±0.24

* E = Estradiol benzoate in corn oil—1 μg./day.
** R = Chilcott Releasin 31-81D SF.
Data of Day Eight
$X = 100$ (dose per day).
$Y = 0.66\,(X - 1.57) + 0.68$.
Doses from 0.10 to 0.80 G.P.U./day.

DISCUSSION

One of the major advantages of employing mice for the bioassay of relaxin, aside from the obvious advantage of housing small animals, is that the animals are used only once and then discarded. In this way the sensitivity of the assay animal tends to be constant and the sensitizing effects of previous courses of estrogen and relaxin are avoided. The effect of employing the same animals for more than a single assay was effectively demonstrated when a group of nine animals were given the usual assay treatment of six days pre-treatment with estrogen plus two days estrogen and relaxin at 0.5 G.P.U. per day. The mean pubic separation increased from 0.33 mm. ±0.02 to 0.86 mm. ±0.15. After a five day rest period these animals were again used. This time the mean pubic separation was

0.84±0.17 at zero hours and increased to 1.44±0.24 at the end of 48 hours. This tendency to increased sensitivity to estrogen and relaxin following a previous pregnancy or assay use has been observed by various workers and represents a serious drawback in the conventional guinea pig assay where the animals are used repeatedly without a rest period of at least 7 to 10 days.

Talmage and Hurst (1950) attempted to refine the traditional guinea pig palpation assay by the radiographic measurement of the interpubic distance, but apparently the guinea pig pelvis separates less than 50 per cent over the resting gap as determined by these workers radiographically. According to their published figures the average interpubic distance determined before relaxin administration among their assay animals was between 0.75 and 1.0 mm. Following the injection of relaxin, there was an average increase of approximately 0.5 mm., but the great variability of response not only between individual animals, but between similar doses in the same animal, was so large that these observers concluded at least 50 animals at each of three dose levels would be necessary to provide a valid assay. They also observed a failure to return to the pre-relaxin measurement in about 10 per cent of pigs used for repeated assays, and a significant number of pigs repeatedly employed for relaxin assay sooner or later became permanently relaxed and had to be discarded.

The superiority of mice as compared to guinea pigs for relaxin assay is undoubtedly related to the dramatic separations which can be produced in the mouse by artificial means. Although we were unable to completely reproduce the separations observed in normal pregnant mice, this objective was more nearly attainable in mice than in guinea pigs. When 10 G.P.U. of relaxin were administered daily in two divided doses for eight days, the pubic gap increased from 0.29 mm. ±0.02 to 2.49±0.30. This was the maximum increment in pelvic gap produced by relaxin, and represented more than 800 per cent of the resting gap. It is probably true that the pubic separation occurring in pregnant mice undoubtedly represents an optimal hormonal environment culminating in a peak separation at term. Hall (1946) has shown that injections of estrone and/or progesterone into intact pregnant mice do not increase the observed separation.

In most of the experiments presented in this paper the mice were selected for assay on the basis of body weight. These animals were therefore of different ages and in order to determine the effect of age upon the individual sensitivity to relaxin, several experiments were performed with groups of animals of various known ages (30 to 73 days). No consistent effect attributable to age could be ascribed and at this time the effect of age prior to ovariectomy cannot be evaluated.

Recent work has been with unoperated intact immature female animals.

The pre-relaxin baseline separation in these animals, as compared with ovariectomized mice, was relatively more constant and only an occasional animal was discarded because the gap measured over 0.5 mm. This assay technique is apparently practical and although the maximum response seems less than with ovariectomized animals, an attempt is being made to determine its possible value.

SUMMARY

A sensitive, convenient and practical bioassay for relaxin is presented employing mice, with radiographic measurement of the pubic separation.

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BIOCHEMICAL PROPERTIES OF FRACTIONS OBTAINED FROM RAT ANTERIOR PITUITARY GLANDS BY DIFFERENTIAL CENTRIFUGATION[1]

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THE biochemistry of the nuclear, mitochondrial, microsomal and supernatant fractions isolated by differential centrifugation from rat and mouse liver has been studied extensively during the past few years. Particular attention has been given to the distribution of the oxidative enzymes and nucleic acids among these fractions. Respiratory enzymes such as cytochrome oxidase and the succinoxidase system have been found to be associated with the mitochondrial fraction (Schneider, 1946; Hogeboom *et al.*, 1946 and 1948). The microsomal fraction contains few enzymes but as much as 50% of the pentosenucleic acid of liver tissue (Hogeboom *et al.*, 1948; Schneider *et al.*, 1950.) The results of studies of this nature have been summarized by Schneider and Hogeboom (1951).

Little attention has been given to the biochemical and hormonal properties of fractions of endocrine tissues isolated by differential centrifugation. Approximately 60% of the succinoxidase activity of rat anterior pituitary glands, however, has been reported to be associated with a large granule fraction (McShan and Meyer, 1949), and 75% of the gonadotrophic hormone activity with a small granule fraction (McShan and Meyer, 1952). The purpose of this paper is to describe a study of the gonadotrophic hormone activity of fractions obtained from anterior pituitary glands of adult normal and castrate rats as related to the succinoxidase, glycolytic and transaminase activities; the desoxypentose-nucleic acid (DNA) and pentosenucleic acid (PNA); and the nitrogen or protein contents of these fractions.

MATERIALS AND METHODS

The anterior pituitary glands of normal and castrate adult female rats of the Holtzman-Rolfsmeyer strain were used for fractionation. The minimum time of castration before using the animals as donors was three weeks and the maximum time was six months.

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The use of rats for experiments of this kind is advantageous as the glands can be removed and homogenized within a few minutes after the animals are killed. This allows little time for tissue changes to occur.

Groups of 100 to 150 rats were used for each run. The pituitary glands were removed in the cold room immediately after killing the animals, and kept in a humidor during the short time required to complete removal of all the glands. The anterior lobes were separated, weighed and macerated in a sharp pointed homogenizing tube with 0.88 M sucrose. The volume of the sucrose in milliliters was 19 times the weight of the glands in grams. The homogenates were prepared by mashing the glands instead of grinding since less gonadotrophin is found in the supernatant fraction when the former procedure is followed (McShan and Meyer, 1952).

The homogenates (WH) were differentially fractionated in the multispeed head of an International refrigerated centrifuge at 2° C. by centrifuging at 275, 4,400 and 20,400 g. to give, respectively, the nuclear (NF), large granule (LG), small granule (SG) and supernatant (S3) fractions according to the procedure of McShan and Meyer (1952). The saline extract of the SG fraction was indicated by SGE and the extracted granules by SGR.

The homogenates and fractions were assayed for gonadotrophic hormone activity by use of 21 day old female rats of the Holtzman-Rolfsmeyer strain. The volumes of the fractions were adjusted with normal saline so that each rat received a total of 20 mg. equivalent of fresh tissue in nine injections of 0.5 ml. each. The first injections were made on the afternoon of the first day followed by injections on the morning and afternoon of the next four days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, and weighed. The presence of follicles and corpora lutea was recorded.

The homogenates and the fractions were stained for mitochondria by use of fast green FCF as reported by Harman (1950) and the nitrogen determinations were done by a modification of the micromethod reported by Johnson (1941).

The procedure for the preparation of filtrates for the nucleic acid determinations was based on the method reported by Schneider (1945a). The exact procedure employed was as follows: The sucrose homogenates and fractions were mixed with one-half volume of 20% trichloracetic acid (TCA), centrifuged at 2,500 RPM in the cold (2° C.) and the supernatant liquid decanted. The precipitates were suspended in 1 to 2 ml. of water to which was added an equal volume of 10% TCA followed by centrifugation. The supernatant liquid was decanted and the precipitates were washed once more with 5% TCA.

The above precipitates were suspended in 1 ml. of water to which was added 3 ml. of 95% ethyl alcohol. This mixture was centrifuged at room temperature followed by decantation of the supernatant liquid, and a second washing with alcohol.

The alcohol precipitates were mixed with 3 ml. of 3:1 ethyl alcohol (95%)—ether mixture, centrifuged and the supernatant liquids decanted. The precipitates were mixed with 2 to 5 ml. of 5 per cent TCA depending on the amount of tissue and heated at 90° C. for 15 minutes, centrifuged and the clear TCA extracts decanted into graduated 15 ml. centrifuge tubes. The precipitates were washed once with 1.5 ml. of 5% TCA, and centrifuged. The washings were mixed with the above extracts and the total volumes were recorded.

The PNA content of the above TCA extracts was determined by the orcinol method of Mejbaum (1939) as recommended by LePage and Umbreit (1945). DNA was determined by the diphenylamine reaction of Dische (1930) as modified by Schneider (1945).

As a basis for calculating the amounts of DNA and PNA present in the homogenized tissues and fractions certain analytical constants for purified samples of these acids were determined according to Schneider (1945a, 1945b). Constants found for the Beckman Model DU spectrophotometer were Fa = 0.01878 (diphenylamine), Fo = 0.1344 (orcinol), and C = 0.01216. These constants were used for calculating the results since the determinations were made with the Beckman instrument. The percentage values used for phosphorus were 0.0954 for PNA and 0.0992 for DNA. A sample calculation using constants for the Evelyn colorimeter is given in the report by McShan, *et al.* (1950).

The succinoxidase was determined by the method of Schneider and Potter (1943), anaerobic glycolytic activity by the method of Novikoff, Potter and LePage (1948), and transaminase activity by the method of Ames and Elvehjem (1946) as modified by Copenhaver, Shipley and Meyer (1951).

RESULTS AND DISCUSSION

The results of the biochemical studies of anterior pituitary tissue from normal and castrate female rats are summarized in Table 1. The gonadotrophic hormone activity of glands from castrate rats was much greater than that of those from normal animals, and the major part of the activity of both kinds of glands was recovered in the small granule (SG) fraction. Essentially all the gonadotrophin in the SG fraction of castrate females was removed by extracting eight times with 0.9% NaCl solution. This extract was designated as SGE. The extracted granules (SGR), and the NF and LG fractions contained little activity whereas the final supernatant fraction S3 contained gonadotrophic activity sufficient to produce ovaries that had an average weight of 55 mg.

Essentially all the DNA of the glands from normal and castrate rats was recovered in the nuclear fraction (NF). The DNA per whole gland was 71.2 μg. for normal and 70.0 μg. for castrate glands (Table 2). On the basis of the assumption that the DNA content of the pituitary cells is constant (Boivin *et al.*, 1948; Swift, 1950), the castrate glands contained about the same number of cells per unit weight as the normal glands. The average weight of the anterior pituitary glands was 10.4 mg. for normal and 11.1 mg. for castrate rats. The ratio of gland weight to weight of DNA per gland was 146 for normal and 159 for castrate animals. This ratio is an expression of relative cell weight and, on the assumption that the specific gravities are the same, it indicates that at least some cells of the castrate glands were larger than those of normal glands. This is supported by the well known fact that the basophils undergo hypertrophy after castration.

The protein values are given in terms of mg. of protein per 20 mg. of fresh tissue (Table 1). These values for protein (N×6.25) are equivalent to the total nitrogen of the various fractions and are, therefore, maximum values since a small amount of nonprotein nitrogen was presumably present. The protein value for WH of glands from normal rats was 15% greater than was that for castrate animals. The values for the LG, SG and

TABLE 1. BIOCHEMICAL PROPERTIES OF FRACTIONS OBTAINED FROM HOMOGENATES OF NORMAL AND CASTRATE ADULT FEMALE RATS

Activity or compound	Units (mg. FT)[1]	Kind of rats[2]	Pituitary fractions								
			WH	NF	LG	SG	SGR	SGE	S3	RCF[3]	Recovery %
Assay—av. wt. ovaries (mg.)[4]	20	N	32 (9)[5]			25 (9)					
	20	C	201(18)	35(6)	36(15)	159(15)	25(17)	145(21)	55(15)		
Protein (N×6.25)	mg./20 mg.	N	3.56(4)[6]	1.03(4)	0.38(4)	0.54(4)	0.23(4)	0.27(2)	1.62(4)		100
		C	3.05(6)	1.01(6)	0.30(6)	0.45(4)	0.20(6)	0.21(6)	1.56(6)		109
Nucleic acid PNA	mg./100 gm.	N	343.6(4)						155.8(5)		
		C	369.4(6)						150.0(6)		
DNA	mg./100 gm.	N	685.2(5)	691.0(6)							101
		C	630.4(5)	602.2(6)							96
Glycolysis	cmm. CO_2/ 20 mg./10 min.	N	25.0(3)	7.0(3)	2.6(3)	1.4(3)			9.5(3)	17.2(3)	82
		C	21.3(3)	5.9(3)	3.1(3)	2.1(3)			(9.8(3)	21.9(3)	103
Transaminase	cmm. CO_2/ 20 mg./10 min.	N	162.0(1)						116.0(1)		
		C	149.4(4)						125.4(4)		
Succinoxidase	cmm. O_2/ 20 mg./10 min.	N	16.3(3)	3.9(3)	4.2(3)	5.4(3)			0.23(3)	16.6(3)	102
		C	14.3(5)	2.7(5)	6.5(5)	1.8(4)			0.50(4)	15.3(4)	107

[1] Milligrams of fresh tissue unless indicated otherwise.
[2] N, normal; C, castrate rats.
[3] Recombined fractions.
[4] Average weight of ovaries from untreated animals of the same age is 13 mg.
[5] Number of animals used for assays.
[6] Number of determinations on which the average value is based.

TABLE 2. DESOXYPENTOSE NUCLEIC ACID CONTENT OF NORMAL AND CASTRATE RAT ANTERIOR PITUITARY GLANDS

Kind of rats	Normal	Castrate
DNA per 100 gm. tissue (mg.)	685.2(5)[1]	630.4(5)
Weight of gland (mg.)	10.4(768)[2]	11.1(958)
DNA per gland (μg.)	71.2	70.0
Gland weight (μg.)/DNA per gland (μg.)	146.0	159.0

[1] Number of determinations on which average value is based.
[2] Number of rats on which average is based.

SGE fractions of glands from normal animals were slightly greater than the values for these fractions of glands from castrate rats. More extractable protein (SGE) was associated with the SG of normal than with those of castrate glands, but the SGR fractions of both normal and castrate glands contained essentially the same amount of protein, namely, 0.23 mg. and 0.20 mg., respectively. Results given in Table 3 show that the protein per gland and the relative amount per cell recovered in the LG, SG and SGE fractions were slightly greater for normal than for castrate glands even though the SG and SGE fractions from the castrate glands contained much more gonadotrophic activity. This means that per unit weight of protein the small granule fraction of glands from castrate rats was much richer in gonadotrophin than the same fraction from glands of normal animals.

The fact that only a slight increase occurred in the total PNA on castration (Table 1) indicates, according to Caspersson (1947), little increase in pituitary protein during castration. Synthesis of small amounts of protein for purposes such as the production of gonadotrophin could have conceivably occurred. However, the possibility should not be overlooked that on castration gonadotrophin was formed from protein already present. It is not clear, of course, as to whether the gonadotrophin was produced by the particules with which it was associated or was concentrated in these particles after production at some other point in the cell.

Essentially 50% of the PNA of the homogenates was recovered in

TABLE 3. CONCENTRATIONS OF PROTEIN IN FRACTIONS FROM NORMAL AND CASTRATE RAT ANTERIOR PITUITARY GLANDS

Compound	Kind of rats	Fraction						
		WH	NF	LG	SG	SGR	SGE	S3
Protein per gland (μg.) (N×6.25)	N	1850	540	198	280	120	140	840
	C	1690	560	166	250	116	115	866
Protein per gland (μg.)[1]	N	26.0	7.58	2.78	3.93	1.72	1.97	11.80
DNA per gland (μg.)	C	24.7	8.00	2.40	3.60	1.66	1.64	12.40

[1] Relative measure of protein per cell.

the S3 fraction. PNA was also found in each of the other fractions but these data are not considered to be reliable from the quantitative standpoint because small amounts of tissue were used for the determinations and the recoveries were not satisfactory. These data are not included. The PNA values for the homogenates were lower than found previously for aqueous homogenates of pituitary glands from young adult rats.

The glycolytic activity of the homogenates of glands from normal rats was slightly greater than the activity of those of castrate rats (Table 1). The NF of both kinds of glands contained more activity than the LG and SG fractions. Almost 50% of the glycolytic activity was found in the supernatant fraction S3 of both the normal and castrate glands which is similar to the results reported by LePage and Schneider (1948) for the soluble fraction of rabbit liver. The SG fractions from both normal and castrate glands were low and not significantly different in glycolytic activity whereas these fractions from normal glands were low and those from castrate glands were high in gonadotrophic activity.

A high percentage of the transaminase activity from both kinds of glands was recovered in the S3 fraction.

The succinoxidase activity of the homogenates of normal glands was 16.3[2] as compared with 14.3 for castrate glands. Satisfactory recovery of activity was obtained when the various fractions were recombined as shown by values of 16.6 and 15.3, respectively, for normal and castrate glands. The NF, LG and SG fractions obtained from both kinds of glands by the procedure referred to above (1952) contained succinoxidase activity. This suggested that these fractions also contained mitochondria and this was shown to be the case by staining. The number present in each of these fractions was not estimated quantitatively. The impression was gained from the staining that mitochondria of varying sizes were present. The fact that they were found in the fractions recovered at different speeds of centrifugation is also indicative of this.

The succinoxidase value for the SG fraction of normal glands was three times the value for castrate glands, namely, 5.4 to 1.8 (Table 1). The latter value for glands of castrate rats is an average of three very low and one high value. The difference between these two values for succinoxidase activity is probably not of any particular significance.

The fact that the LG fraction contains little gonadotrophic activity indicates that gonadotrophin is not associated to a significant degree with the mitochondria of this fraction. On the basis of this it was suggested (McShan and Meyer, 1952) that the gonadotrophin of the SG fraction was not associated with the mitochondria of this fraction, but this explanation does not seem valid since it is conceivable that the gonadotrophin could be associated with the small mitochondria or with other granules contained in the SG fraction. It was reported previously (McShan and Meyer, 1952) that little gonadotrophin is associated with the material recovered from the S3 fraction of an 0.88 M sucrose homogenate by centrifuging at 150,000 g. which, according to the work of Hogeboom, Schneider and Pallade (1948), would be expected to contain submicroscopic particles.

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[2] Cu. mm. of oxygen uptake per 20 mg. of fresh tissue per 10 minutes.

SUMMARY AND CONCLUSIONS

Anterior pituitary glands from normal and castrate adult female rats were homogenized in 0.88 M sucrose and separated by differential centrifugation into nuclear, large granule, small granule and supernatant fractions. The gonadotrophic activity, the protein, PNA and DNA contents, and the glycolytic, transaminase and succinoxidase activities of the homogenates and fractions of both kinds of glands were studied.

The high level of gonadotrophin found in the castrate as compared with the normal glands was concentrated in the small granule fraction, and it was removed from this fraction by extraction with isotonic saline. The distribution of protein in the two kinds of glands was studied. The granule fractions and the extract of the small granules of the castrate glands contained slightly less protein than the same fractions of normal glands.

The DNA was recovered in the nuclear fraction and approximately 50 per cent of the PNA in the supernatant fraction. Castration caused little change in the DNA and PNA.

The glycolytic activity was essentially the same for the homogenates and fractions obtained from glands of normal and castrate rats, but the gonadotrophic hormone activity was much greater in the small granule fraction of glands from castrate as compared with normal animals. The major part of the transaminase was found in the supernatant fraction. The succinoxidase was distributed in the nuclear and granule fractions of both kinds of glands. These fractions as indicated by staining were found to contain mitochondria.

On the basis of these results and those reported previously it appears that though gonadotrophin is not associated to a significant degree with the mitochondria of the large granules nor with the submicroscopic particles recovered by high speed centrifugation of the supernatant fraction, it may be associated with those of the small granules.

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RESPONSE OF PREPUTIAL AND ADRENAL GLANDS OF HYPOPHYSECTOMIZED RATS TO ACTH[1]

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THE growth response of the preputial glands of the female rat to ACTH in adrenalectomized-ovariectomized animals indicates that these glands are subject to the direct action of this trophic hormone (Jacot and Selye, 1952). Furthermore, it has been shown that the ascorbic acid of male rat preputial glands can be depleted by stress or ACTH in acute experiments, and that these alterations in the vitamin content resemble the changes which occur in the adrenal gland following these same stimuli (Hess *et al.*, 1952).

It has been known for some time that the level of adrenal ascorbic acid is greatly reduced by hypophysectomy (Leblond and Chamorro, 1940; Bowman *et al.*, 1940). Tyslowitz (1943) reported that the concentration of ascorbic acid in the adrenal glands gradually declines for about 18 days following the operation. Thereafter, he found that the concentration remained stabilized at about 60% of the value for intact animals. To date, there is no information concerning the concentration of ascorbic acid in the preputial glands of the hypophysectomized rat. The present studies were undertaken to determine the effect of hypophysectomy, together with acute and chronic administration of ACTH, on the weight and ascorbic acid content of the preputial glands, as compared to the adrenal glands of the rat.

METHODS

Twenty-nine male rats of the Holtzman strain were hypophysectomized at the age of 33 days. During the postoperative period they were maintained on laboratory chow and 5% sucrose solution. At the time of autopsy the sella turcica was examined for hypophyseal fragments with the aid of a dissecting microscope. The absence of any such fragments, together with a sustained loss of body weight and a marked loss of testis and ventral prostate weight was considered satisfactory evidence of the completeness of the operation.

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Experiment I. At the end of 3 weeks following hypophysectomy the left preputial and left adrenal glands were removed from 14 animals under nembutal anesthesia. Ten of the animals were then injected with 10 μg. ACTH,[3] via the tail vein, and after one hour the right preputial and right adrenal glands were removed. The glands were freed of excess adipose and connective tissue, weighed on a torsion balance, and subjected to ascorbic acid analysis in accordance with the method of Roe and Kuether (1943).

Experiment II. Two weeks after hypophysectomy, the left preputial and left adrenal glands were surgically removed from 15 animals. Following this operation, the animals were given subcutaneous injections of 25 μg. ACTH twice daily for 7 days. At autopsy the right preputial and right adrenal glands were removed, weighed and analyzed for ascorbic acid content.

Twenty-three intact animals of the same age and sex were used as controls for these experiments. In addition to weights and ascorbic acid values for preputial and adrenal glands, weights for the left testis and ventral prostate were obtained from these animals.

RESULTS

Experiment I. Three weeks after hypophysectomy there is an absolute as well as a relative decrease in the weight and ascorbic acid content of the preputial and adrenal glands (Table 1). On a relative weight basis the

TABLE 1. THE EFFECT OF HYPOPHYSECTOMY AND ACTH TREATMENT ON THE ADRENAL AND PREPUTIAL GLANDS OF MALE RATS

			Adrenal Glands				Preputial Glands			
	No. of Rats		Weight		Ascorbic Acid Content		Weight		Ascorbic Acid Content	
			Abs	Rel.[2]	Abs.[3]	Rel.[4]	Abs.	Rel.[2]	Abs.[3]	Rel.[4]
Control	10	L	mg. 13.9 ±0.30*	mg. 8.1 ±0.26	60 ±2.6	439 ±18	mg. 39.3 ±5.0	mg. 22.7 ±2.8	22 ±3.3	57 ±6.3
	10	R	13.1 ±0.22	7.6 ±0.20	60 ±2.2	456 ±16	32.4 ±4.4	18.6 ±2.4	20 ±3.1	59 ±6.8
Hypophysect. (3 weeks)	14	L	5.4 ±0.18	5.1 ±0.19	13 ±0.6	247 ±11	9.7 ±0.5	9.1 ±0.5	3 ±0.9	24 ±7.2
	10	R[5]	4.8 ±0.22	4.4 ±0.20	8 ±1.0	170 ±20	10.5 ±0.9	9.8 ±0.9	1 ±0.3	6 ±2.8
Hypophysect. (2 weeks)	15	L	5.6 ±0.19	5.3 ±0.25	19 ±1.7	348 ±26	11.0 ±0.7	10.5 ±0.7	3 ±1.1	21 ±9.1
Hypophysect. (3 weeks) plus ACTH[1]	15	R	6.1 ±0.28	5.8 ±0.30	17 ±0.8	276 ±12	15.4 ±0.9	14.5 ±0.8	4 ±1.6	29 ±10.3

[1] 25 μg. ACTH injected subcutaneously twice daily for 7 days prior to autopsy.
[2] mg./100 gm. body weight.
[3] μg. of ascorbic acid in the entire gland.
[4] μg. ascorbic acid/100 mg. tissue.
[5] 10 μg. ACTH injected intravenously 1 hr. prior to autopsy.
* Standard error.

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[3] Armour ACTHAR. (dosage expressed as LA-1A equivalent)

preputial and adrenal glands of the hypophysectomized rat are 60% and 37% smaller, respectively, than those of control animals. Likewise, the concentration of preputial and adrenal ascorbic acid is 58% and 44% less than that of the normal glands. In spite of the fact that the concentration of ascorbic acid is greatly reduced in these glands following hypophysectomy, they are still capable of responding to an intravenous injection of 10 μg. of ACTH by a further reduction in their ascorbic acid content (Table 1). Thus a 75% depletion of preputial and a 31% depletion of adrenal ascorbic acid was seen at one hour following the injection of ACTH.

Experiment II. The 7-day treatment with ACTH resulted in some enlargement of the preputial glands as seen by comparing their weights with those of uninjected hypophysectomized rats (Table 1). Likewise, there was a slight enlargement of the adrenal glands, although it was considerably less than was anticipated. It is clear that ACTH reverses the gradual decline in the weight of the preputial and adrenal glands seen at 2 and 3 weeks after hypophysectomy. These injections, on the other hand, had only little effect on the preputial and adrenal ascorbic acid concentrations, the values being similar to those observed in the uninjected, hypophysectomized animals.

It has been the general observation in this study that left preputial and adrenal glands are slightly heavier than right ones. This was true in the intact control group, where on a mg./100 gm. body weight basis, the left preputial gland was 18% heavier than the right and the left adrenal gland was 6% heavier than the right (Table 1). There was no significant difference, however, in the concentration of ascorbic acid in the right and left glands. In the two hypophysectomized groups, since the right glands were utilized for study after the administration of ACTH, either acutely or chronically, it is not known whether this same tendency prevails. If one assumes that left glands tend to be heavier than right ones in untreated, hypophysectomized rats, it follows that in the present study the preputial and adrenal hypertrophy produced by chronic ACTH administration is partially masked.

DISCUSSION

Following hypophysectomy there is a gradual decline both in the weight and ascorbic acid content of the preputial and adrenal glands. The results for the adrenal glands are in general agreement with the earlier findings of Tyslowitz (1943). The decline proceeds at a more rapid rate in the preputial glands where it is apparently complete at 2 weeks after hypophysectomy. It is of considerable interest that, in spite of the marked involution of the preputial and adrenal glands and their low levels of ascorbic acid, they are still sensitive to ACTH, as indicated by the further depletion of ascorbic acid following the acute administration of 10 μg. ACTH. In comparison to the adrenal gland, the preputial gland is apparently more sensitive to the ascorbic acid depleting action of ACTH.

Although the administration of ACTH for one week to animals which had been hypophysectomized 2 weeks previously was capable of reversing the gradual decline in preputial and adrenal weight, it had little effect in rebuilding the ascorbic acid content of these glands. The concentration of preputial and adrenal ascorbic acid is not significantly different from the values for the untreated, 3-week hypophysectomized animals. In a previous study of this sort Munson and Koch (1946) reported that 13 daily injections of ACTH to hypophysectomized rats raised the ascorbic acid content of their adrenal glands. Their dosage range was from 40 μg. to 1 mg. daily and they state that even when sufficient ACTH was given to increase the adrenal weight above that of unoperated controls, the adrenal ascorbic acid was still significantly less than that of the controls. It seems clear that the action of ACTH on adrenal as well as on preputial gland ascorbic acid is more complex than is indicated by the depletion phenomenon which rapidly follows the administration of a single injection. In the absence of further information it is suggested that ACTH may also influence the synthesis of ascorbic acid so that with the chronic administration of this hormone to hypophysectomized animals a new equilibrium between synthesis and utilization of ascorbic acid in these glands is reached. If this is true, it appears that at the dosage employed the activity level is well below that of the glands of intact animals, as indicated by the relatively low levels of glandular ascorbic acid. Further indication of low activity level is the relatively slight effect on the preputial and adrenal gland weight produced by the dosage of ACTH employed. Apparently the dosage of ACTH employed was near the minimally effective dosage of the preparation used.

It has been known for some time that the preputial glands of the rat are subject to androgenic stimulation (Korenchevsky, 1935). That this is not a factor in the present study is indicated by the pronounced atrophy of the testes and ventral prostate in both groups of hypophysectomized animals (Table 2). The preputial glands are also responsive to some degree to the action of estrogen and progesterone (Van der Woerd, 1938) and are currently regarded as accessory reproductive glands. Developmentally they are modified sebaceous glands and functionally they belong to the holocrine type, their secretory product being a viscous sebum. By usual histological and cytological criteria they have little in common with the adrenal cortex. It is therefore somewhat surprising that these glands show such a striking parallelism to the adrenal glands in their response to ACTH. So far as is known at present, the direct action of ACTH is limited to the adrenal cortex and the preputial glands. Long (1947) has shown that changes in ascorbic acid levels of other organs such as brain, kidney, heart,

TABLE 2. THE EFFECT OF HYPOPHYSECTOMY AND ACTH TREATMENT ON THE BODY, TESTIS, AND PROSTATE WEIGHT OF RATS

	No. of Rats	Body weight		Left testis weight		Ventral prostate weight	
		Initial	Final	Abs.	Rel.[2]	Abs.	Rel.[2]
		gm.	gm.	mg.	mg.	mg.	mg.
Control	13	120 ±0.9*	179 ±2.9	1114 ±34	628 ±16	128 ±18	71 ±8.9
Hypophysect. (3 weeks)	14	122 ±1.7	109 ±1.8	206 ±12	191 ±11	9.5 ± 0.6	8.8 ±0.5
Hypophysect. (3 weeks) plus ACTH[1]	15	123 ±1.3	106 ±1.5	202 ±15	189 ±13	9.1 ± 0.8	8.6 ±0.8

[1] 25 μg. ACTH injected subcutaneously twice daily for 7 days prior to autopsy.
[2] mg./100 gm. body weight.
* Standard error.

skeletal muscle and lymphoid organs are of an insignificant nature following a single injection of ACTH. The marked differences together with the marked similarities between the preputial and adrenal glands offer the promise that further studies of this nature will shed new light on the role of ascorbic acid in cellular processes.

SUMMARY

Hypophysectomy of male rats produces a gradual decline in both weight and ascorbic acid content of the preputial and adrenal glands. At three weeks postoperatively when the concentration of ascorbic acid is at a low level in both of these glands it can be further reduced by a single intravenous injection of ACTH. A 7-day treatment, begun two weeks postoperatively reverses the gradual decline in the weight of the preputial and adrenal glands but has little effect on their ascorbic acid content. As in intact animals, there is a close parallelism in the response of the preputial and adrenal glands to ACTH.

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ENDOCRINE INFLUENCE ON THE PLASMIN-PLASMIN INHIBITOR SYSTEM IN THE BLOOD OF RATS

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INTRODUCTION

EARLIER studies have indicated that the equilibrium of the plasmin (fibrinolysin)-plasmin inhibitor (antifibrinolysin) system in blood can be shifted under various experimental conditions (Jensen, Chamovitz, Volkringer, Gray, Gosney and Kocholaty, in press). Ungar and his associates (1951, 1951) reported that certain endocrine principles act on this system by accelerating the rate of combination of plasmin and plasmin inhibitor. The findings of the present study do not seem to substantiate this concept. Instead, the recorded data tend to show that the increased antifibrinolytic activity, observed after administration of certain hormones, is a result of an actual rise in the plasmin inhibitor level in the blood.

MATERIALS AND METHODS

Male Sprague-Dawley albino rats weighing 220–250 gm were used. The animals were allowed food and water throughout the experiment. Unless noted all injections were given subcutaneously. Controls received either no injections or a volume of solvent equal to that used in the hormone injections.

Splenectomy was performed on fasting rats anesthetized by ether. Animals regained their normal weight within the second or third post-operative day. The experimental work was carried out 7 days after splenectomy. Hypophysectomized and adrenalectomized rats, obtained from the Hormone Assay Laboratories, Inc., Chicago, Illinois, were used 10 days after operation.

The hormones were injected subcutaneously in dosages as follows:

Adrenocorticotropin, Armour Acthar, equivalent to 1.0 mg Standard LA-1-A/kg twice daily for 3 days; controls—equivalent volume of 0.9% saline.

Cortisone, Merck Cortone Acetate, 5 mg/kg daily for 3 days; controls—equivalent volume of 0.9% saline.

Thyroxine, Squibb Crystalline Thyroxine, 0.5 mg/kg daily for 4 days; controls—equivalent volume of slightly alkaline 0.9% saline.

Thyrotropin,[1] Armour Lot 2-D-3, LJW3-16-48 containing 5 Junkmann-Schoeller units per mg, 10 mg/kg daily for 5 days; controls—equivalent volume of slightly alkaline 0.9% saline.

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[1] This preparation was kindly supplied by the Armour Laboratories, Chicago, Illinois.

Blood samples, 1.8 ml, were taken by heart puncture 2 hours after the last injection, using 0.2 ml of 3.8% sodium citrate as the anticoagulant. Following centrifugation at 2800 rpm for 30 minutes at 5 to 10°C, the plasma was separated and either analyzed immediately or stored frozen until tested.

The antifibrinolytic activity of plasma was determined by measuring the residual activity of a standard amount (2 mg[2]) of bovine plasmin,[3] after incubation for 5 and 30 minutes with a 4% concentration of plasma as previously described by Westphal, Kocholaty, McDonald, King and Jensen (1950). Thrombin, salt-free, (Parke-Davis) and fibrinogen, prepared according to Ware, Guest and Seegers (1947), were used. Calculations of antifibrinolytic units (AFU) were performed as previously reported by Kocholaty, Ellis and Jensen (1952).

The plasmin inhibitor preparation used in these studies was obtained by $(NH_4)_2SO_4$ fractionation of a product prepared from bovine plasma according to Loomis, Ryder and George (1949). The plasmin inhibitor was found to be precipitated between 0.6–0.7 saturation of ammonium sulfate.

RESULTS AND DISCUSSION

When the plasmin inhibitor preparation was tested against plasmin, it was found that 340γ of this preparation were equivalent to one antifibrinolytic unit. Tested against trypsin, 60γ corresponded to one unit. This finding is in agreement with earlier observations of Christensen and MacLeod (1945) and of Lewis and Ferguson (1950) who found that the inhibition of plasmin requires a higher concentration of the serum or plasma than is necessary to inhibit trypsin of equal fibrinolytic potency.

Table 1. Inhibition of bovine plasmin by normal rat plasma

0.1 ml (2000γ) of bovine plasmin incubated for 5 and 30 minutes at 25° C. with 0.1 ml of rat plasma ranging from 0.5–6% in the final reaction mixture. After addition of thrombin (0.1 ml, 10 units) and fibrinogen (0.2 ml, 500γ), the clot dissolution time was determined at 37.5° C. Imidazole buffer pH 7.25 was used.

Plasma concentration (%) in the reaction mixture	CDT (Seconds)*	
	5 minute incubation	30 minute incubation
0.0	123	126
0.5	129	136
1.0	135	147
1.5	145	160
2.0	153	175
2.5	166	195
3.0	182	216
3.5	203	249
4.0	234	305
4.5	275	355
5.0	314	420
5.5	382	528
6.0	455	610

* Each clot dissolution time (CDT) is the average of duplicate determinations.

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[2] This amount of plasmin dissolved the standard fibrin clot in 125 seconds at 37.5°C.

[3] Preparations of bovine plasmin were kindly supplied by Dr. E. C. Loomis of the Research Laboratories, Parke-Davis and Company, Detroit, Michigan.

The rate of combination between plasmin and its inhibitor is dependent on the time of incubation of the reactants; a 30 minute incubation produced a greater inhibition than did the 5 minute incubation, both in the case of rat plasma and of a purified preparation of bovine plasmin inhibitor (Table 1 and 2). These findings are in agreement with similar observations reported by Guest, Daly, Ware and Seegers (1948). It was found that the inhibition of trypsin by normal rat plasma after 30 minutes incubation was somewhat greater than after 5 minutes incubation, but the difference was not as pronounced as that observed with plasmin. As to be expected, the rate of combination between plasmin and its inhibitor was found to be dependent also on the temperature of the reaction mixture.

TABLE 2. INHIBITION OF BOVINE PLASMIN BY PLASMIN INHIBITOR

0.1 ml (2000γ) of bovine plasmin incubated for 5 and 30 minutes at 25° C. with 0.1 ml containing 20–140 of purified bovine plasmin inhibitor. After addition of thrombin (0.1 ml, 10 units) and fibrinogen (0.2 ml, 500γ) the clot dissolution time was determined at 37.5° C. Imidazole buffer pH 7.25 was used.

Plasmin inhibitor Gamma	CDT (seconds)*	
	5 minute incubation	30 minute incubation
0	126	129
20	130	138
40	131	145
60	134	154
80	137	163
100	139	166
120	142	177
140	143	193

* Each clot dissolution time (CDT) is the average of duplicate determinations.

The antifibrinolytic activity of normal rat plasma after 5 minutes incubation was 87.6 ± 7.5 units, after 30 minutes, 105.8 ± 5.8 units (Table 3). The plasmas of control rats injected either with saline or alkaline saline had similar antifibrinolytic activities. The administration of adrenocorticotropin over a 3-day period in normal rats produced a significant increase in the antifibrinolytic activity of the blood, as measured after 5 or 30 minutes incubation, 101.4 ± 13.5 units and 119.3 ± 8.5 units, respectively, (Table 3). Thyrotropin, given for 5 days, produced an increase noted only after 5 minutes incubation. Thyroxine, administered for 4 days, produced an elevation of the antifibrinolytic activity which was evident only after 30 minutes incubation while cortisone, given for 3 days, produced no change.

Single intraperitoneal injections of adrenocorticotropin (1 to 4 mg. per rat), of adrenal cortical extract, Upjohn, (2 to 4 ml. per rat) and of thyroxine (1 mg. per rat) were found to produce no significant change in the plasmin inhibitor titer 15 to 90 minutes after administration.

The antifibrinolytic activity of plasma 10 days after removal of the hypophysis or of the adrenals was found to be greatly reduced (Table 3). Splenectomy did not alter the plasmin inhibitor level of plasma (Table 3). In splenectomized rats, adrenocorticotropin administration (same procedure as in normal animals) did not produce any change in the antifibrinolytic activity of the blood, as measured after 5 or 30 minutes incubation (Table 3). Apparently, the influence of the adrenals on the plasmin inhibitor titer is mediated through the spleen. In this connection it may be pointed out that Macfarlane and Biggs (1948) have observed that among all the organs, extracts of the spleen had the highest antifibrinolytic activity. On the other hand, administration of thyroxine (same procedure as in normal animals) to the splenectomized rat produced a significant elevation of the antifibrinolytic titer as measured after 30 minutes incubation (Table 3). Apparently, more than one mechanism is involved in the control of the plasmin inhibitor level in blood.

TABLE 3. EFFECT OF REMOVAL OF ENDOCRINE ORGANS AND OF VARIOUS HORMONES ON THE ANTIFIBRINOLYTIC ACTIVITY OF RAT PLASMA

Treatment	No. of animals	Incubation time					
		5 Minutes			30 Minutes		
		CDT[1] Avg. Sec.	AFU[2]/ml plasma Avg. ±S.D.	t^3	CDT Avg. Sec.	AFU/ml plasma Avg. ±S.D.	t
Normal Animals							
No treatment	13	234	87.6 ± 7.5		284	105.8 ± 5.8	
Saline	10	238	88.5 ± 9.3		295	108.8 ± 7.6	
Alkaline Saline	10	234	87.6 ± 8.3		291	107.9 ± 9.0	
ACTH‡	15	274	101.4 ± 13.5	2.8†	339	119.3 ± 8.5	3.2‡
Cortisone	15	240	88.9 ± 12.8	0.9	311	112.7 ± 9.7	1.1
Thyroxine	15	231	85.3 ± 8.6	0.4	318	114.9 ± 6.2	2.2*
TSH	15	255	95.6 ± 8.3	2.6†	311	113.0 ± 7.7	1.5
Hypophysectomy	4				202	60[4]	
Adrenalectomy	4				230	76[4]	
Splenectomy[5]							
Saline Controls	3	239	89.0		284	107.0	
ACTH	9	254	94.3 ± 12.7	1.1	298	109.7 ± 7.7	0.3
Thyroxine	9	242	90.9 ± 8.1	1.1	327	117.1 ± 6.9	2.5*

[1] Clot dissolution time.

[2] Antifibrinolytic units.

[3] Comparisons are made with the injected controls. p values: * = <0.05, † = <0.02, ‡ = <0.01.

[4] Tested against standard trypsin after 30 minutes incubation only. The normal value with trypin—104 AFU/ml of plasma.

[5] Previous studies showed no changes in the antifibrinolytic titer after splenectomy.

In general, these findings are in agreement with the observations of Ungar and coworkers (1951) namely, an increased antifibrinolytic activity after the administration of adrenocorticotropin to normal animals and no alteration in the plasmin inhibitor titer when administered to splenectomized animals. However, the results presented do not seem to substantiate the concept suggested by these workers that adrenocorticotropin affects the antifibrinolytic activity of normal plasma by accelerating the rate of combination between plasmin and plasmin inhibitor. Instead, the recorded data tend to show that the observed increased antifibrinolytic activity is a result of an actual rise in the plasmin inhibitor level in the blood.[4]

SUMMARY

The degree of combination between plasmin and plasmin inhibitor was found to depend on the incubation time and temperature of the reactants.

Administration of adrenocorticotropin produced a significant increase in the plasmin inhibitor titer of blood. The injection of thyrotropin or thyroxine caused elevations of the plasmin inhibitor in blood. Cortisone produced no change. The plasmin inhibitor titer of rat plasma 10 days after removal of the hypophysis or of the adrenals, was found to be greatly reduced. Splenectomy did not alter the plasmin inhibitor level of plasma. In splenectomized rats adrenocorticotropin administration did not produce any change in the plasmin inhibitor titer of the blood. On the other hand, administration of thyroxine to splenectomized rats was found to produce an elevation of the titer.

The significance of these findings has been discussed.

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[4] It may be pointed out that the observed increase in antiplasmin activity can also be caused by a decrease in the plasmin of the blood. The test, as employed, does not differentiate between fibrinolytic and antifibrinolytic activity.

NOTES AND COMMENTS

THE INFLUENCE OF CORTISONE ON THE IODIDE CONCENTRATING MECHANISM OF THE RAT THYROID*

Several groups of workers have found a depression of thyroid I^{131} uptake in cortisone-treated rats (Money *et al.*, 1951, Verzár and Vidovič, 1951, Perry, 1951, Albert *et al.*, 1952). An inhibition of the pituitary thyrotrophic mechanism was ruled out as the cause of this phenomenon by the findings that the rate of release of radioiodine from the thyroid is not reduced by cortisone (Perry, 1951, Albert *et al.*, 1952) and that the morphology of the thyroid in cortisone-injected rats suggests a compensatory hypersecretion rather than a diminished output of thyrotrophin (TSH) (Halmi and Barker, 1952). Albert *et al.* (1952), in a trenchant analysis of the cortisone-induced changes in thyroid function, pointed out that reduced I^{131} uptake may be the result of various disturbances, such as an *increased renal elimination of iodide, an augmentation of the iodide space* or an inhibition of the concentration of iodide by the thyroid. One might add to this list an interference with the organic binding of iodine in the thyroid.

The present study was undertaken to ascertain the effect of large doses of cortisone on the ability of the thyroid to concentrate iodide. Since Woodbury *et al.* (1951) reported that exogenous TSH is less effective in raising the thyroidal I^{131} uptake of hypophysectomized rats if cortisone is administered simultaneously, we also investigated the influence of cortisone on the thyroid iodide trap of hypophysectomized rats receiving TSH. Finally we examined the effect of cortisone on the rise in the thyroid: serum iodide ratio which follows the administration of goitrogens (Vanderlaan and Vanderlana, 1947).

MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain were used in this study. In the first experiment 10 rats were injected with daily doses of 8 mg of cortisone[1] for 10 days. Ten uninjected control rats were pair-fed. In the second experiment hypophysectomized rats (100–150 gm.) were obtained 8 days after operation from the Hormone Assay Laboratories, Chicago and divided into 3 groups. The first group received no treatment, animals in the second group were injected with 1 mg. of TSH (USP Thyrotropin Reference Substance) daily for 10 days, while to those of the third group 1 mg. of TSH and 5 mg. of cortisone were administered daily over a period of 10 days. In the third experiment 10 rats received 10 daily injections of 10 mg. of propylthiouracil in an alkaline solution, while 10 pair-fed rats were given 10 mg. of cortisone daily for 10 days in addition to the same amount of propylthiouracil. All unoperated rats received powdered Rockland chow, while the hypophysectomized animals were given the same diet in pellet form and supplemented with horse meat and oranges.

The iodide concentrating ability of the thyroids was determined by the method of Vanderlaan and Greer (1950). All rats were injected subcutaneously with 5 mg. of propylthiouracil in an alkaline solution. Forty-five minutes later they received an injection of 5 μc. of carrier-free radioiodide.[2] One or two hours following the latter injection the rats were anes-

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* Aided by a grant from the Central Scientific Fund, College of Medicine, State University of Iowa.

[1] Cortisone (Cortone acetate Merck) was generously supplied by Dr. E. Alpert, Merck and Co., Rahway, N. J.

[2] Radioiodide was obtained through the courtesy of Dr. T. C. Evans, Radiation Research Laboratory, State University of Iowa.

thetized with ether and blood was withdrawn from the abdominal aorta. The thyroids were dissected out, weighed on a torsion balance and squashed on a piece of filter paper, which was placed on an aluminum planchet and covered with scotch tape. One tenth of a ml. of serum was pipetted onto a piece of filter paper of the same size, which then was also fixed to a planchet with scotch tape. The radioactivity of the thyroids and the blood samples was determined by means of a thin mica-window Geiger-Müller counter. Since the propylthiouracil almost completely prevents the organic binding of the subsequently administered I^{131} (Vanderlaan and Vanderlaan, 1947), the radioactivity of the thyroid can be regarded as the measure of radioiodide collection by the gland. The thyroid:serum iodide ratio is expressed as the quotient of the respective activities of 100 mg. of thyroid tissue and 0.1 ml. of serum.

RESULTS

Table 1 shows that the thyroid:serum iodide ratio in rats treated with 8 mg. of cortisone daily for 10 days was somewhat higher than that of pair-fed controls. Table 2 summarizes the observations on groups of hypophysectomized rats which received a) no treatment, b) 1 mg. of TSH daily for 10 days, and c) 1 mg. of TSH and 5 mg. of cortisone daily for 10 days. The low thyroid:serum iodide ratio found in the untreated hypophysectomized rats is in agreement with the findings of Greer (1949). TSH markedly elevated the thyroid:serum iodide gradient and cortisone did not exert any significant influence on this response to TSH. The results of the third experiment, which are shown in Table 3,

TABLE 1. THYROIDAL IODIDE CONCENTRATING ABILITY OF RATS GIVEN 8 MG. OF CORTISONE DAILY FOR 10 DAYS AND OF THEIR PAIR-FED CONTROLS

Group	No. of rats	Average Starting weight gm.	Average Final weight gm.	Thyroid: serum iodide ratio (mean ±st. error)	
Cortisone	10	194	153	27.9±2.6	P=0.02
Control	10	210	213	20.8±0.4	

TABLE 2. THYROIDAL IODIDE CONCENTRATING ABILITY OF GROUPS OF HYPOPHYSECTOMIZED RATS RECEIVING NO TREATMENT, 1 MG. OF TSH DAILY FOR 10 DAYS OR 1 MG. OF TSH+5 MG. OF CORTISONE DAILY FOR 10 DAYS

	No treatment	TSH only	TSH+cortisone
No. of rats	14	13	13
Thyroid: serum iodide ratio (mean±st. error)	5.0±0.7	32.9±2.6	30.2±2.6
		P=0.4	

TABLE 3. THYROIDAL IODIDE CONCENTRATING ABILITY OF RATS RECEIVING 10 MG. OF PROPYLTHIOURACIL+10 MG. OF CORTISONE DAILY FOR 10 DAYS AND OF PAIR-FED CONTROLS GIVEN 10 MG. OF PROPYLTHIOURACIL DAILY FOR 10 DAYS

Group	No. of rats	Average Starting weight gm.	Average Final weight gm.	Thyroid: serum iodide ratio (mean ±st. error)	
Propylthiouracil+cortisone	10	195	152	92.7±15.7	P=0.7
Propylthiouracil only	10	199	193	101.8± 6.4	

indicate that the simultaneous administration of 10 mg. of cortisone and of 10 mg. of propylthiouracil daily failed to prevent the rise in the thyroid:serum iodide ratio which was elicited by 10 daily doses of 10 mg. of the latter drug alone.

DISCUSSION

The results presented in this paper indicate a) that the defective thyroid I^{131} uptake observed by others in cortisone-treated rats is not likely to have been the sequel of an interference of this steroid with the ability of the thyroid to concentrate iodide, b) that the partial inhibition exerted by cortisone upon the TSH-induced rise in thyroid I^{131} uptake of hypophysectomized rats (Woodbury *et al.*, 1951) is equally unlikely to have been the result of interference with the iodide trapping mechanism, and c) that, indeed, a striking elevation of the thyroid: serum iodide ratio in response to a goitrogen is compatible with the simultaneous injections of large amounts of cortisone. The fact that cortisone-treated rats had an apparently slightly higher thyroid:serum iodide ratio than that of their pair-fed controls is in agreement with the concept that this steroid is not a TSH inhibitor, since the thyroid:serum iodide gradient drops if TSH does not act on the thyroid. Furthermore, it harmonizes with histological findings indicating an increased thyrotrophic stimulation of the thyroids of cortisone-treated rats (Halmi and Barker, 1952). Since both the pituitary thyrotrophic mechanism and the thyroid iodide trap thus appear to be ruled out as points of attack of cortisone, the following hypotheses remain to be verified or refuted: a) cortisone affects the extrathyroidal metabolism of iodide (renal excretion, iodide space), b) it partially inhibits a phase of the organic binding of iodine in the thyroid.

SUMMARY

Cortisone did not materially affect the iodide trapping ability of the thyroids of normal rats, of hypophysectomized rats given thyrotrophin, or of rats chronically treated with propylthiouracil.

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The author is much indebted to the donors, to the several physicians who drew the human blood samples and to Dr. Gertrude van Wagenen, Department of Obstetrics and Gynecology, Yale University, and her assistants who obtained blood samples from monkeys in Dr. van Wagenen's colony.

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THE USE OF COLLODION ULTRAFILTRATES OF HUMAN URINE FOR STEROID DETERMINATIONS

INTRODUCTION

The practicability of determining the content of more than one hormone in a single sample of human urine is limited, essentially, by the concentration of the desired hormones in the urine. Thus, the gonadotrophins, pregnanediol and the adrenal cortical steroids are usually present in such small amounts, particularly in non-pregnant subjects, that quantities of urine varying from about $\frac{1}{5}$ of a day's output to as much as a 48 hour output, or more, may be required for the determination of only one of these. It is often desirable, however, from the standpoint of the patient's convenience, as well as the research objective, to know the excretion level of the gonadotrophins and of a steroid hormone or its metabolites during a particularly limited period of time, such as 24 or 12 hours. Our use of the Gorbman (1945) method for extracting gonadotrophins by ultrafiltration through a collodion membrane suggested that a simple solution to this problem might be at hand if the ultrafiltrate could be used for the steroid assays. This has been tested by determination of the 17-ketosteroid and pregnanediol content of several urines before and after filtration through collodion. We have found that, with certain reservations, the technique is a practical one, and, to our knowledge, has not been reported before.

METHODS AND MATERIALS

A total of 39 complete 24 hour urines were studied for total 17-ketosteroid, and 26 urines were studied for pregnanediol content before and after ultrafiltration. The urines were collected from a group of young healthy female inmates of a home for the feeble minded. All urines were kept refrigerated, with thymol as the only preservative, until used.

Gonadotrophins were extracted on aliquots of each urine, as described by Gorbman (1945). In the pregnanediol series, a single aliquot of urine, equal to $\frac{1}{5}$ of the day's output, was first removed, the remaining $\frac{4}{5}$ of the day's urine ultrafiltered, and pregnanediol determined on a single $\frac{1}{5}$ aliquot of the ultrafiltrate, and on the aliquot of unfiltered urine. For the 17-ketosteroid series, a 10.0 cc. volume of unfiltered urine was first removed, the remainder of the urine ultrafiltered, and the 17-ketosteroid determinations carried out on a 10.0 cc. volume of the ultrafiltrate and the 10.0 cc. aliquot of unfiltered urine. The method of Sommerville, Gough and Marrian (1948) was used for pregnanediol, and that of Drekter, *et al.* (1947) for 17-ketosteroid analyses.

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TABLE 1. TOTAL 17 KETOSTEROID CONTENT OF HUMAN URINE BEFORE AND AFTER ULTRAFILTRATION THROUGH COLLODION

The values are presented in three groups: (A) urines containing less than 10.0 mg./24 hours; (B) urines containing between 10.1 and 15.0 mg./24 hours; and (C) urines containing more than 15.0 mg./24 hours. The values shown in the first two columns of each group are mg. of 17-ketosteroid per 24 hours. The group means for the percentage change from the value of the urine before filtration are derived from the difference between the before and after filtration group means, rather than from the sum of the individual percentages. There is a slight discrepancy between these two values only in the case of the first group of urines (less than 10.0 mg./24 hours).

A. Less than 10.0 mg. per 24 hours			B. 10.1 to 15.0 mg. per 24 hours			C. More than 15.0 mg. per 24 hours		
Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*
4.9	5.8	+18	10.1	7.8	−23	15.1	13.9	−7.9
5.0	6.3	+26	10.8	8.0	−26	15.8	14.6	−7.6
6.3	5.2	−17	10.9	8.3	−24	16.0	16.0	0
6.4	9.5	+48	11.2	9.7	−13	16.0	16.0	0
6.6	8.0	+21	11.4	9.5	−17	16.6	15.3	−7.8
6.9	6.0	−13	12.6	12.6	0	17.3	14.7	−15
7.2	7.9	+9.7	13.1	13.1	0	17.5	17.0	−2.9
7.3	4.8	−34	13.2	10.5	−20	20.4	19.9	−2.4
9.2	7.0	−24	13.4	15.3	+14	20.9	14.4	−31
9.4	11.4	+21	15.0	15.0	0	21.6	19.6	−9.3
9.5	8.0	−16				21.9	21.9	0
9.8	7.2	−27				22.0	21.2	−3.6
9.9	6.6	−33				22.6	22.6	0
						25.5	25.2	−1.2
						26.5	21.4	−19
						48.6	50.3	+3.5
Group means:								
7.6	7.2	−5.3	12.2	11.0	−9.9	21.5	20.2	−6.1
Mean of % change, regardless of sign:		23.7			13.7			6.9

* From the value of the urine before filtration.

RESULTS

17-ketosteroids. The 17-ketosteroid values, in terms of mgs./24 hours, are shown in Table I. The values are arranged in three groups, according to the quantity of steroid excreted per day, in order to show its relation to the percentage change (expressed in all cases in relation to the amount present in the urine before filtration) following filtration. There was a very obvious inverse relation between the daily level of 17-ketosteroid excretion and the amount of variation in the values of the percentage change following ultrafiltration. This is shown by the decrease in the value of the mean of the percentage change, *calculated without regard to the sign of this change* (shown at the bottom of each group of values). The true means showed little difference between the three groups of urines, and the fact that most of the minus changes were found in the intermediate and high content urines is an indication of a probable slight but real loss of steroid as a result of filtration.

Pregnanediol. Not as many urines were examined for pregnanediol content, and the values, though extending over as wide a relative range as did the 17-ketosteroids, did not compare with the latter as far as absolute amounts are concerned. Nevertheless, very much the same sort of relationship was seen (Table 2), and what has been said about the

TABLE 2. PREGNANEDIOL CONTENT OF HUMAN URINE BEFORE AND AFTER ULTRAFILTRATION THROUGH COLLODION

The values presented are in two groups: (A) urines containing less than 1.0 mg./24 hours; and (B) urines containing more than 1.0 mg./24 hours. As in Table 1, the values in the first two columns of each group are mg. of pregnanediol per 24 hours, and the group means for the per cent change are derived from the difference between the before and after filtration group means.

A. Less than 1.0 mg. per 24 hours			B. More than 1.0 mg. per 24 hours		
Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*
0.17	0.25	+47	1.17	0.90	−23
0.19	0.09	−52	1.35	0.44	−67
0.20	0.20	0	1.37	1.19	−13
0.25	0.20	−20	1.38	1.47	+6.5
0.26	0.28	+7.7	1.62	1.41	−13
0.26	0.22	−15	1.70	1.48	−13
0.28	0.23	−18	1.70	1.77	+4.1
0.28	0.39	+39	1.77	1.55	−12
0.36	0.58	+61	1.89	1.68	−11
0.40	0.25	−37	1.99	1.90	−4.5
0.74	0.55	−26	2.07	2.34	+13
0.86	0.13	−85	2.42	2.23	−7.9
0.96	0.21	−78	4.90	7.50	+53
Group means:					
0.40	0.27	−32	1.95	1.97	−1.03
Mean of % change, regardless of sign		37%			19%

* From the value of the urine before filtration.

size of the change following filtration and the amount of 17-ketosteroid in the urine, applies in general as well to the pregnanediol series.

DISCUSSION

When one considers that the two values compared in this study were obtained from single aliquots of urine, the amount of variation between them is not surprising and should not be disturbing. For clinical purposes there can hardly be any question of the value of using the ultrafiltrate of urine for a steroid determination, since the alternative of using separate 24 hour collections will be associated with variations at least as large as those resulting from the passage through collodion. In addition, the importance of such determinations for clinical purposes is not obviated by a loss or gain from the real value of roughly 20%. However, the difference from the true value as a result of filtration may be serious in certain areas of research where relatively small differences in output are anticipated, but this is to some extent minimized by the fact that the percentage change is more frequently a large one almost only when one is dealing with relatively small concentrations of steroid. If this is recognized, and data interpreted accordingly, there is no reason why the method cannot be used, especially if the determinations are made on at least duplicate aliquots of the ultrafiltrate. It has thus been of inestimable help in allowing us to perform a single experiment to determine simultaneously the effect of progesterone on gonadotrophin output, and the conversion of progesterone to pregnanediol in relation to the phases of the menstrual cycle.

SUMMARY

The 17-ketosteroid and pregnanediol content of human urine after ultrafiltration through collodion is shown to vary from the content of these steroids in the unfiltered urine within limits small enough to allow the ultrafiltrate to be used for the determination of excretion levels of these substances.

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THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.

THE AMERICAN GOITER ASSOCIATION

1953 ANNUAL MEETING

The 1953 Annual Meeting will be held May 7, 8 and 9, 1953, in the Drake Hotel, Chicago, Illinois. The program for the three day meeting will consist of papers and discussions dealing with goiter and other diseases of the thyroid gland.

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COMPARATIVE EFFECT OF ACTH, CORTISONE AND DCA ON RENAL FUNCTION, ELECTROLYTE EXCRETION AND WATER EXCHANGE IN NORMAL DOGS

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THE inadequacies of our knowledge of the role of adrenocortical hormones in the regulation of electrolyte and water metabolism are becoming increasingly apparent (Gaunt, *et al.*, 1949; Ingle, 1950; Sayers, 1950). Extensive and numerous observations on the action of DCA have been reported (Ragan, *et al.*, 1940; Ferrebee, *et al.*, 1941; Mulinos, *et al.*, 1941; Zierler and Lilienthal, 1948; Gaudino and Levitt, 1949; Davis, *et al.*, 1951; Luft and Sjögren, 1952; Sjögren, 1952), but data on the effect of 11,17-oxysteroid hormones in the dog are meager (Thorn, *et al.*, 1941; Davis, *et al.*, 1951; Roberts and Pitts, 1952). The latter studies were with cortisone and observations with ACTH are necessary before adequate evaluation of the relation of the ACTH-adrenocortical system to salt metabolism can be made. An important consideration which has been neglected in most studies with ACTH and cortisone in the dog and rat is the role of glomerular filtration rate in the regulation of salt and water excretion. As demonstrated in this report, data on alterations in the rate of glomerular filtration are essential for satisfactory evaluation of electrolyte excretion during hormone administration.

The present study was undertaken to help clarify the relation of adrenocortical hormones to salt and water metabolism. The salt retaining effects of ACTH, cortisone, and DCA have been compared and the relation of glomerular filtration rate to changes in electrolyte excretion and water

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exchange has been studied. Since the response to adrenocortical hormones differs among species, the results in the dog have been compared with those obtained previously in man (Zierler and Lilienthal, 1948; Daughaday and MacBryde, 1950; Alexander, *et al.*, 1951; Ingbar, *et al.*, 1951; Luft and Sjögren, 1952; Sjögren, 1952) and rat (Rice and Richter, 1943; Ingle, *et al.*, 1946; Bergner and Deane, 1948; Boss, *et al.*, 1950).

MATERIALS AND METHODS

ACTH, cortisone, and DCA were administered to four normal female mongrel dogs. After a 2 to 3 week control period, these hormones were given successively for intervals of 5 to 31 days; a recovery period was allowed following each hormone injection period. A saline solution of ACTH[1] was injected intramuscularly every six hours whereas saline suspensions of cortisone acetate and DCA in oil were given intramuscularly once daily. Approximate doses of 2.1 and 6.3 mgm./kg./day of ACTH and 2.1 and 4.2 mgm./kg./day of cortisone were employed for dogs 1 and 2, but dogs 3 and 4 received the larger dose only (Figures 1–4). All four animals received approximately 1.0 mgm./kg./day of DCA. Two levels of sodium (Na) intake (0.5 and 3.3 m.Eq./kg./day) were given during cortisone administration to dog 3 and during injection of all three hormones in dog 4; otherwise, observations were made during the higher level of Na intake.

Glomerular filtration rate (GFR) and effective renal plasma flow (RPF) were determined in the postabsorptive state by measuring the renal clearances of creatinine (C_{CR}) and para-aminohippurate (C_{PAH}) respectively. Renal clearances were measured from 3 to 5 hours after hormone administration. During the renal function studies, blood was drawn for determination of plasma electrolytes and arterial plasma carbon dioxide content. T-1824 dye space was measured and circulating eosinophils enumerated. Femoral arterial blood pressure was determined following the renal clearance procedure.

The dogs were kept in metabolic cages except during experimental procedures. Sodium, potassium (K), chloride (Cl), nitrogen (N), and water balance studies were made according to methods reported previously (Davis and coworkers, 1952). A synthetic diet consisting of 80 Cal./kg./day was force fed so that intake was constant except during vomiting or parenteral administration of additional electrolytes. Water was allowed *ad libitum.*

The experimental procedures and chemical methods have been described elsewhere (Davis, *et al.*, 1952) with these exceptions: (1) plasma Cl in dogs 3 and 4 and the Cl content of food and feces were determined by a modified Volhard technique, (2) the carbon dioxide content of arterial plasma was measured manometrically by the method of Van Slyke and Neill (1924), and (3) for the eosinophil counts, a mixture of propylene glycol and phloxine was used as a diluent (Speirs and Meyer, 1949).

RESULTS

Alterations in renal hemodynamic function were qualitatively similar for all three hormones (Figs. 1–3). The response consisted of an increase in GFR and RPF while filtration fraction (FF) fell. Renal blood flow was also elevated since hematocrit remained at the control level. The magnitude of the effect varied considerably among animals as well as with the hormone administered. The response was greatest with cortisone, least with DCA, and moderate with ACTH. The most marked elevations occurred in dogs 1 (Fig. 1) and 3 in which renal function increased after a single injection of each hormone. The least response resulted in dog 2 (Fig. 2) in which renal function was not detectably altered during ACTH administration, and a slight elevation occurred only after several days of cortisone or DCA. In dog 4, GFR and RPF were increased after a single injection of ACTH and cortisone; no apparent difference was observed between the effect on low and high Na intake. DCA failed to increase renal function in dog 4 even after prolonged administration on a high Na intake.

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[1] The ACTH was the ACTHAR product of Armour Laboratories; each unit was equivalent to 1 mgm. of the lyophilized standard LA-1-A.

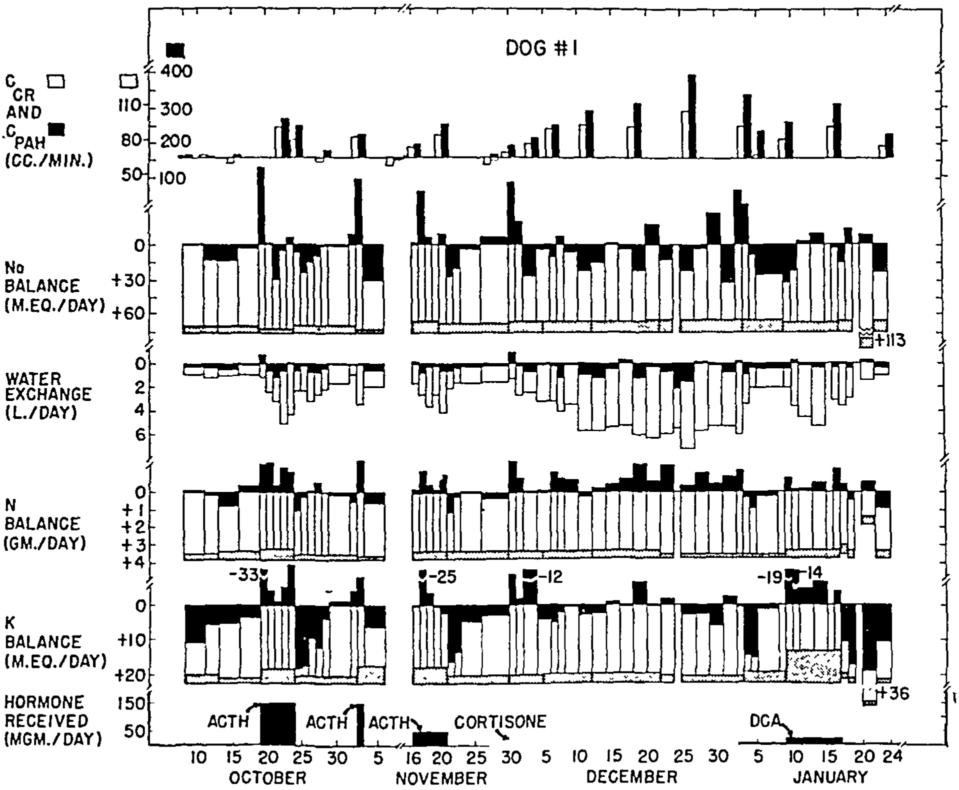

FIG. 1. The effect of ACTH, cortisone, and DCA on renal function and metabolic balances in a 24 kg. dog. Values for C_{CR} and C_{PAH} were plotted as open and solid columns respectively from a horizontal line which represents the control level. Each value is the average of 3 postabsorptive clearance periods. The convention for the balances is as follows: (1) intake was plotted downward from the zero line, (2) output was plotted upward from intake line, (3) solid black areas represent total balances, and (4) the cross-hatched areas show fecal excretion. Consequently, solid black area above the zero line indicates negative balance and black area below the zero line represents positive balance.

Another striking effect of these hormones was the increase in water exchange. The typical response (3 of 4 dogs) to ACTH and cortisone was an increase in water turnover from about $\frac{1}{2}$ to approximately 2 L./day (Figs. 2 and 3) but in dog 1 water exchange reached 6 to 7 L./day (Fig. 1). In this dog, the magnitude of water turnover showed a high positive

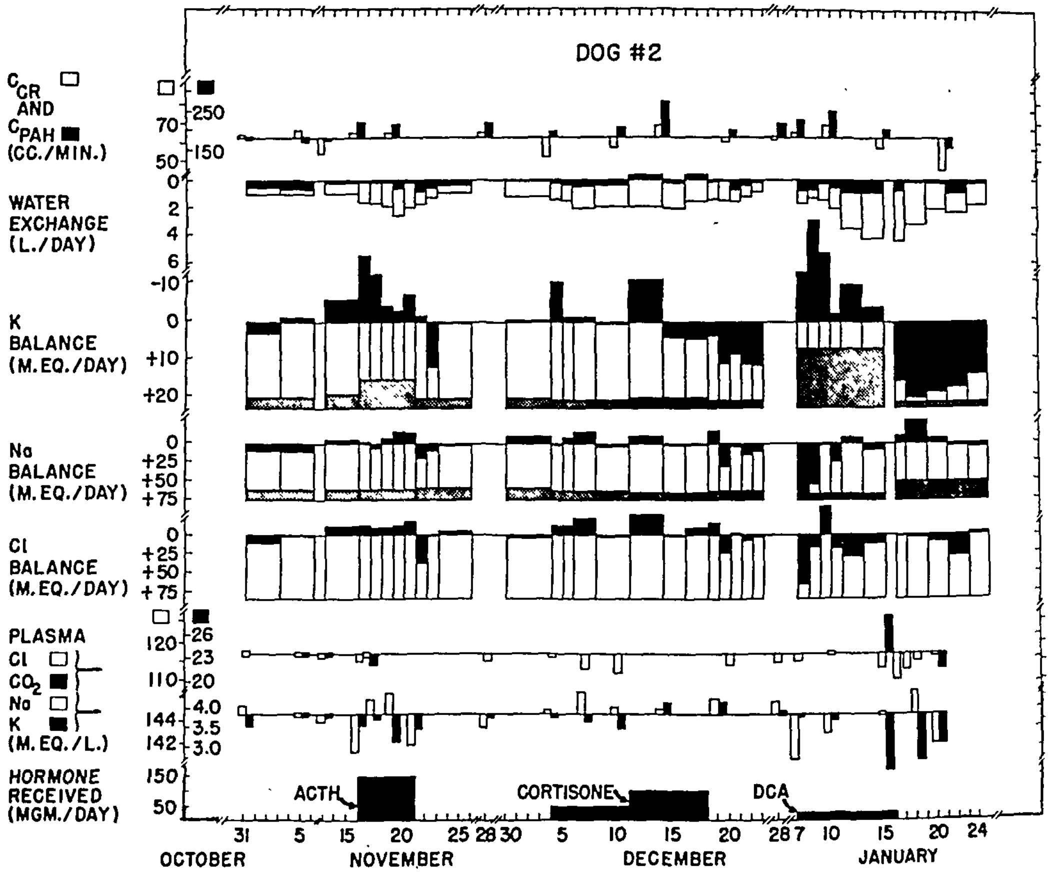

FIG. 2. Alterations in renal function, water exchange, electrolyte balances, and plasma electrolytes during ACTH, cortisone, and DCA administration to a 24 kg. dog. The horizontal line from which values for plasma electrolytes are plotted represents the average control value. See Figure 1 for the convention for the balance plots and other symbols.

correlation with the level of GFR for all three hormones and the onset of polyuria preceded the development of polydipsia. The response to DCA was less variable among the animals and a diabetes insipidus-like state occurred in all cases on a high salt diet (Figs. 1–3). The level of water exchange was independent of Na intake during cortisone administration (cases 3 and 4; see Fig. 3), but DCA had no influence on water turnover on a low Na intake (dog 4) (Fig. 3). The effect of Na intake on water exchange during ACTH administration was equivocal (Fig. 3).

The first 1 or 2 days of ACTH and cortisone injections were accompanied by salt loss in dogs 1, 3, and 4; GFR was elevated initially in these three dogs but not in dog 2 (Figs. 1–3). Salt retention resulted during the corresponding period on DCA. Thereafter, Na and Cl balance was maintained on ACTH, cortisone, and DCA although wide daily fluctuations in Na and Cl excretion occurred during prolonged cortisone administration (Fig. 1). Fecal Na excretion was unaffected by ACTH and cortisone but DCA effected a slight depression which was followed by an elevation during the recovery period in cases 2 (Fig. 2) and 3.

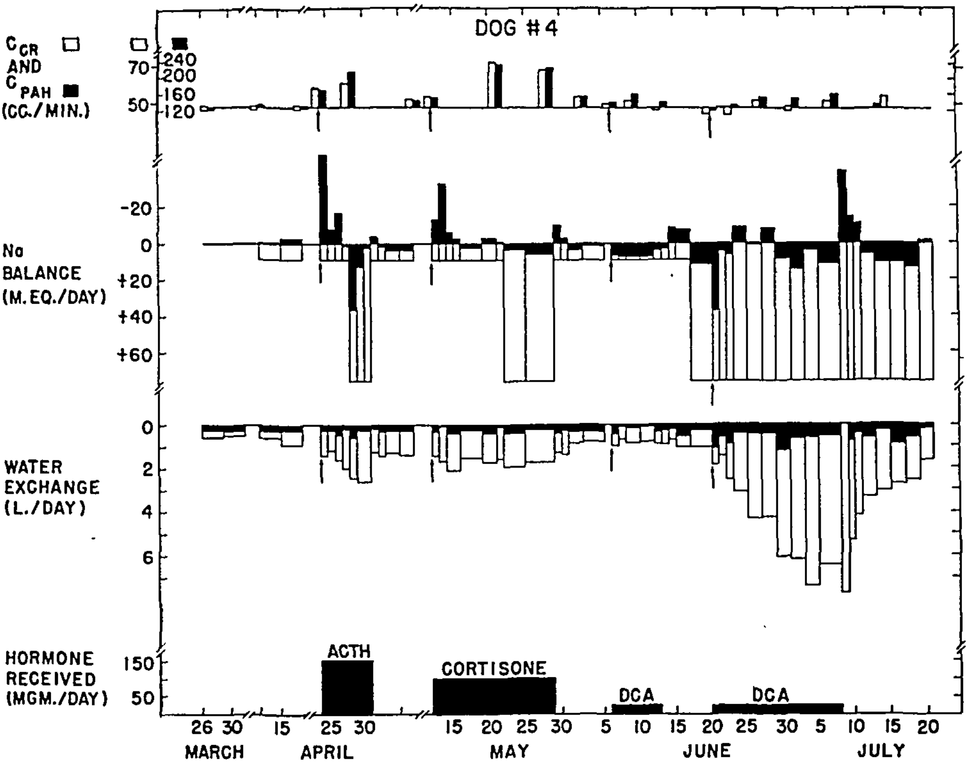

FIG. 3. Renal function, Na balance, and water exchange in a 22 kg. dog given ACTH, cortisone, and DCA during a low and a high Na intake. For a description of symbols and convention for the balances, see Figure 1.

Urinary K excretion increased initially with all three hormones during high salt intake; later, the influence of cortisone became negligible and K loss during ACTH and DCA diminished (Figs. 1, 2, and 4). In dog 4, the initial loss of K also occurred on a low salt diet with ACTH, but cortisone and DCA failed to produce a negative K balance unless salt intake was high. The greatest increase in fecal K output accompanied DCA but an elevation also occurred with ACTH except in case 3; cortisone was without effect.

Negative K balance was most marked with DCA which resulted in a metabolic alkalosis in all instances except during low salt intake in dog 4 (Figs. 1, 2, and 4). After 8 to 10 days of DCA injections, dogs 1 and 2 exhibited signs of K deficiency including generalized muscle weakness, nystagmus, lassitude, abdominal distention, and vomiting. In contrast, dog 4 was given DCA for 18 days without clinical evidence of K deficiency although plasma K dropped to 1.9 m.Eq./L. (Fig. 4). Plasma K and water exchange became progressively more abnormal as long as DCA was given, but in dog 4 plasma CO_2 reached a maximum after 11 days and remained unchanged until DCA was discontinued. Hypochloremia occurred during the DCA alkalosis in dogs 1–3 (see Fig. 2) but not in dog 4. Plasma Na was elevated during DCA administration in all but dog 2. ACTH and cortisone did not detectably alter plasma electrolytes.

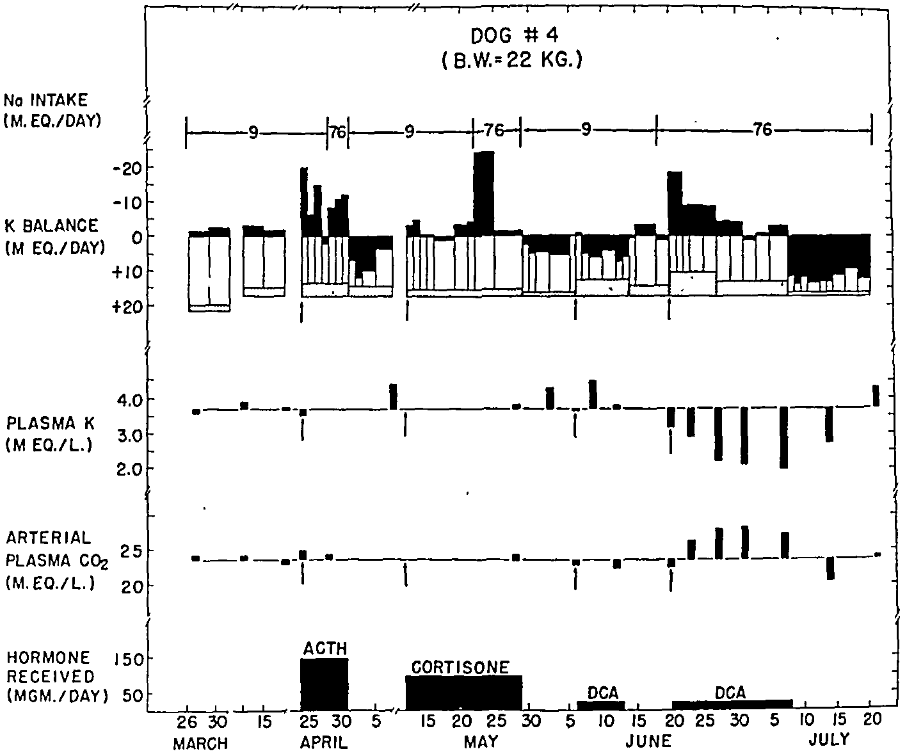

FIG. 4. Potassium balance, plasma K, and arterial plasma CO_2 during ACTH, cortisone, and DCA administration on a low and a high Na diet. Values for plasma K and arterial plasma CO_2 were plotted from a horizontal line which represents the control level. The convention for K balance has been given with Figure 1.

A negative nitrogen balance occurred throughout ACTH and cortisone administration but the only consistent effect of DCA was increased N excretion on the first day of injection. Circulating eosinophils decreased or disappeared during injection of ACTH and cortisone in dogs 1, 2, and 4; in dog 3, repeated studies before and after ACTH administration failed to reveal a decline in eosinophils and the effect of cortisone was less than in the other cases. T-1824 dye space, hematocrit, plasma proteins, and femoral arterial blood pressure were not detectably changed. Heart rate increased only during the alkalosis with DCA.

DISCUSSION

The typical response in the renal circulation to ACTH, cortisone, and DCA was an increase in the flow of blood through the kidney. Renal arteriolar dilatation was indicated by the finding of elevated renal blood flow while arterial pressure remained at the control level. The response in renal function was observed from 3 to 5 hours after a single injection for all three hormones in dogs 1 and 3. This observation may indicate that the initial rise in renal function was effected by an action on the renal arterioles. The sustained elevation in renal function may also have resulted in part from a direct renal effect. On the basis of available data, the response to ACTH and cortisone cannot be related to expansion of the extracellular fluid volume as has been suggested for DCA by Gaudino and Levitt (1949) since the initial effect of ACTH and cortisone was a natriuresis and balances for salt and water were never positive. However, the possibility that ACTH and cortisone can effect a shift in fluid from the intracellular to the extracellular compartment as reported by Levitt and Bader (1951) has not been excluded.

The pattern of renal functional changes in the dog differs slightly from that found for man (Alexander, *et al.*, 1951; Ingbar, *et al.*, 1951). In both species, GFR and RPF may be elevated by ACTH and cortisone but in man FF is usually increased whereas in the dog it is reduced. In the rat, no significant alteration in GFR was noted during injection of cortisone (Cotlove, unpublished data), but DCA has been reported to decrease GFR slightly (Boss, *et al.*, 1950); these studies were not accompanied by measurements of RPF.

Water exchange was increased by all three hormones but the phenomenon produced by DCA appeared to differ from that with ACTH and cortisone both quantitatively and qualitatively. A diabetes insipidus-like state occurred with DCA only, except in dog 1 in which water exchange was markedly elevated with both ACTH and cortisone. In this animal the high positive correlation between the level of GFR and water exchange may indicate that GFR played a role in the development of the diabetes insipidus-like state. Also, in dog 1 the primary polyuria during both ACTH and cortisone administration may be a reflection of the initial elevation in GFR. During injection of cortisone, water turnover was unaffected by changing the level of Na intake, whereas, as shown previously by Mulinos and associates (*1941*) for the dog and by Rice and Richter (1943) for the rat, the level of water exchange during DCA is a function of salt intake. In man, the level of water turnover is uninfluenced by ACTH, cortisone, or DCA (Prunty, *et al.*, 1948; Zierler and Lilienthal, 1948; Daughaday and MacBryde, 1950; Sprague, *et al.*, 1950; Alexander, *et al.*, 1951; Sjögren, 1952).

Distortion of the plasma electrolyte pattern and development of alkalosis which frequently results during ACTH or cortisone administration in man (Prunty, *et al.*, 1948; Sprague, *et al.*, 1950; Sjögren, 1952) failed to occur in the dog. The duration of ACTH administration may have been too short to result in alkalosis, but the dose and duration of cortisone injection were sufficient to demonstrate a negative result. The negative K balance which occurred during ACTH and cortisone was probably a reflection of increased tissue catabolism since a concomitant loss of N resulted. The data indicate that the marked K depletion which accompanied DCA resulted from a different mechanism. It seems unlikely that K loss and alkalosis during DCA administration were a passive consequence of Na retention as has been proposed by Seldin and coworkers (1951) since Na retention lasted from 1 to 2 days only whereas a negative K balance was present throughout DCA administration. A more plausible explanation is that K depletion was the primary event. Under this circumstance, it has been suggested by Cooke, *et al.* (1952), that hydrogen ions move intracellularly with Na in exchange for K and result in alkalosis. Also, K ions may be less available for distal tubular exchange for Na than normally so that sufficient hydrogen ion secretion by the kidney occurs to prevent disappearance of the alkalosis (Berliner, Kennedy, and Orloff, 1951). Failure of alkalosis to develop with DCA during low salt intake has been reported previously by Seldin and associates (1951) and is apparently related to inadequate anion excretion. Excessive loss of K by the fecal route may indicate a direct influence of adrenocortical hormones on the intestinal mucosal transport of electrolytes (Dennis and Wood, 1940).

The effect of adrenocortical hormones on salt excretion also varies among species. An initial natriuretic and chloruretic action has been reported during administration of ACTH to man (Forsham, *et al.*, 1948) and rat (Ingle and associates, 1946; Bergner and Deane, 1948), but the salt retention which follows the initial period in man has not been observed in the rat. In the present study in the dog, both ACTH and cortisone produced an initial salt loss and, like the effect in the rat, Na retention did not occur later. It has been suggested that the negative balances for Na and Cl in man resulted from a temporary increase in 11,17-oxysteroid secretion before the effect of DCA-like hormones became manifest (Forsham, *et al.*, 1948). In the dog, it seems more likely that salt loss resulted from elevated GFR; the natriuresis occurred only in the three dogs with an initially increased GFR and not in the other animal in which GFR remained at the control level.

In contrast to the initial effect of ACTH and cortisone on salt excretion in the dog, DCA resulted in Na retention in spite of elevated GFR in two of the four dogs. This finding indicates that the Na retaining effect of DCA is greater than that of cortisone or adrenocortical hormones released by ACTH at the dosage levels employed. The effect of DCA on electrolyte metabolism appears to be similar in dog and man except that negative Na and Cl balances have been reported in man following prolonged (34–60 days) administration (Sjögren, 1952).

After the initial response, salt balance was maintained during administration of all three hormones. GFR was frequently elevated so that increased tubular reabsorption of Na and Cl can be inferred in these instances. It is not certain from these data whether ACTH stimulated secretion of hormones promoting Na and Cl reabsorption by the renal tubules or if salt balance was achieved by another mechanism. A salt retaining effect of cortisone in the normal dog was likewise equivocal. In the adrenalectomized dog, however, cortisone appears to have a direct renal tubular action to conserve Na. Roberts and Pitts (1952) found a marked reduction in the renal excretion of Na while GFR remained at the control level. Also, adrenalectomized dogs have been maintained in excellent health for several weeks on cortisone alone (Swingle, *et al.*, 1952; Howell and Davis, unpublished data) although a low Na and a high K concentration in the plasma frequently occurred even on high doses of cortisone. The finding that a low plasma Na was associated with a negative Na balance (Howell and Davis, unpublished observations) and the present comparative data with DCA suggest that cortisone is a weak Na retaining compound. If ACTH stimulated secretion of adrenocortical salt retaining hormones, failure of salt retention might be an indication that their weak salt retaining action was overbalanced by elevated GFR.

It is evident from the foregoing discussion that the role of the ACTH-adrenocortical system in the regulation of salt metabolism has not been clearly defined. In man, it seems fairly certain that ACTH stimulates secretion of adrenocortical hormones which influence the renal tubular transport of electrolytes (Ingbar and associates, 1951), but in the dog and rat, the situation may differ. In addition to the observations with exogenous ACTH, studies during salt restriction have demonstrated that the response in hypophysectomized dogs differs from that in patients with panhypopituitarism. These patients show impaired ability to conserve

Na; in the report of Stephens (1940), a low plasma Cl (Na not measured) and evidence of acute adrenocortical insufficiency developed within three days. In contrast, the hypophysectomized dogs studied by Earle and coworkers (1951) failed to show a drop in plasma Na during ingestion of a 1 to 2 m.Eq./day Na diet for three weeks. More recently, Rolf and associates (1952) reported that hypophysectomized dogs on a low Na intake lost slightly more Na than normal dogs, but 2 to 5 weeks were required before plasma Na fell. These experiments and studies with exogenous ACTH indicate that anterior pituitary control of adrenocortical salt regulating function differs in dog and man. Additional observations of electrolyte metabolsim in the hypophysectomized dog are needed to determine the exact nature of this difference. Cytochemical studies of the adrenal cortex in the dog under circumstances similar to those reported for the rat (Deane, 1949) may also help to clarify this important aspect of anterior pituitary and adrenocortical physiology.

SUMMARY

The response to ACTH, cortisone, and DCA was studied successively for variable periods in four female mongrel dogs. GFR and RPF increased with all three hormones (except with ACTH in one dog and DCA in another). The immediate effect of these hormones may be a reflection of an action on the renal arterioles. Water exchange was increased by all three preparations. The diabetes insipidus-like state produced by DCA was dependent upon a high Na intake, whereas increased water turnover of lesser magnitude occurred with ACTH and cortisone on both low and high Na diets. A negative K balance resulted with ACTH, cortisone, and DCA but marked K depletion and alkalosis accompanied DCA and a high salt intake only. Fecal K excretion was increased greatly by DCA and slightly by ACTH (3 of 4 dogs) but cortisone was without effect. The first 1 or 2 days of ACTH and cortisone administration were accompanied by salt loss in 3 of the 4 dogs; since GFR was initially increased in these three but not in the fourth animal, a causal relationship between elevated GFR and the negative balance is suggested. Salt retention resulted during the corresponding period on DCA. Thereafter, balance was maintained during administration of all three hormones in spite of a high GFR. Although it is uncertain whether ACTH stimulated secretion of hormones promoting Na and Cl reabsorption by the renal tubules, the effect of ACTH and cortisone on salt excretion in the dog differs from that reported for man.

Acknowledgment

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STUDIES ON THE INTERRELATIONSHIPS OF THE ADRENAL CORTEX AND RENAL AMMONIA EXCRETION BY THE RAT[1]

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IT HAS been demonstrated recently that the renal capacity of the adrenalectomized animal to secrete ammonium and hydrogen ions is deficient under normal conditions and under conditions of moderate to severe acid stress (Sartorius *et al.*, 1952). Failure to salvage adequately fixed base from the tubular urine in exchange for hydrogen or ammonium ions, results not only in increased loss of base but in a disturbance of body acid base balance. These facts probably account for the chronic acidosis and the poor tolerance to acid stress commonly exhibited by animals with adrenal insufficiency.

The present study was undertaken (a) to determine the capacity of normal and adrenalectomized animals to excrete ammonia and titratable acid when acutely subjected to mild and severe acid stress, (b) to assess the effects of desoxycorticosterone acetate and adrenal cortical extract on ammonia and titratable acid excretion by the adrenalectomized and normal rat, (c) to study the early excretory rates of these ion species after adrenalectomy in an effort to separate or to further link the renal tubular exchange mechanisms, and (d) to ascertain whether acidosis stimulates the adrenal cortex directly as evidenced by a lowering of the glandular content of cholesterol and of ascorbic acid.

METHODS

All experiments were performed on female Sprague Dawley rats weighing from 200 to 250 grams. Animals were bilaterally adrenalectomized in one stage and were allowed a recovery period of 7 to 10 days or more, except where otherwise specified. The operative procedure for the sham operated rats was the same as for those adrenalectomized except that following exposure and mobilization the glands were replaced to their normal position. Adrenalectomized rats were maintained on a high salt diet without further supporting therapy. During the course of each experiment, animals were kept in wire mesh metabolism cages, so constructed as to prevent contamination of the urine with food or feces. In all acute experiments urine was expressed from the bladder by slight supra-

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pubic pressure and collected under oil in small-necked containers in order to reduce evaporating surface. Adequate urine flows were obtained in acute experiments by the administration of 2 cc. of distilled water per 100 grams of body weight 15 minutes prior to the experiment. Water or ammonium chloride as a 5% solution was administered by stomach tube. Desoxycorticosterone acetate in sesame oil was injected subcutaneously in dosages of a half a milligram per rat, 18 hours prior to and 1 hour prior to the experiment.

Urine pH was determined with a Cambridge Glass Electrode, titratable acid by electrometric titration of the urine to a pH of 7.40, urinary ammonia by Conway's microdiffusion method (1942), adrenal cholesterol according to the method of Schoenheimer and Sperry (1934), and adrenal ascorbic acid by the method of Roe and Kuether (1943).

RESULTS

In order to quantitate more precisely the relative deficiencies in capacity of the adrenalectomized rat to secrete ammonia and titratable acid, acute experiments were performed on normal, 2 weeks adrenalectomized, and 2 months adrenalectomized rats subjected to mild and severe acidosis. Production of a mild dilution acidosis was accomplished by the administration of water alone in dosage of 2 cc. per 100 grams of body weight. A more severe acidosis was effected by the administration of ammonium chloride in dosage of 100 mg. per 100 grams of body weight dissolved in the same volume of fluid. Following an initial control period of one hour duration the acid load was imposed and the urine collected for four successive hourly periods. The numbers of rats used in any experiment are indicated in parentheses on each chart.

As is evident in Figure 1, normal rats excreted approximately 11.6 microequivalents of ammonia per 100 grams of rat per hour during the control period. With administration of water alone and the consequent production of a mild dilution acidosis, ammonia excretion rose gradually to a peak of 16 microequivalents. With the added stimulus of an acidifying agent, the excretory rate rose sharply, reaching an average level of 74.3 microequivalents for the fifth hour. In sharp contrast with these levels are those of the adrenalectomized animals. From initial control levels of but 6.6 microequivalents of ammonia per 100 grams of rat per hour, there occurred an increase to but 8.9 microequivalents under water loading and a rise to only 41.1 microequivalents with acid loading. It is thus apparent that although the adrenalectomized rat is capable of increasing ammonia output under stimulation, there is a marked deficiency in its capacity to do so. That this deficiency is not dependent upon, or altered by the length of time following operation is suggested by the fact that two weeks post-adrenalectomized rats and the same group six weeks later exhibited practically identical excretory rates for both ammonia and titratable acid.

Titratable acid excretory rates for both normal and adrenalectomized rats are shown in the upper right hand portion of Figure 1. From control rates of less than 0.2 microequivalent excreted per 100 grams of rat per hour, normal rats responded with an increase to 4.1 microequivalents when given a water load and to an average level of 24.4 microequivalents in the fifth hour when acid loaded. A marked deficiency in the capacity of the adrenalectomized rat to secrete hydrogen ions is apparent, for the maximum excretion with water loading was 0.64 microequivalent while with the imposition of an acid load an excretory rate of only 8.4 microequivalents was attained. The higher urinary pH of the adrenalectomized rat likewise reflects this defect.

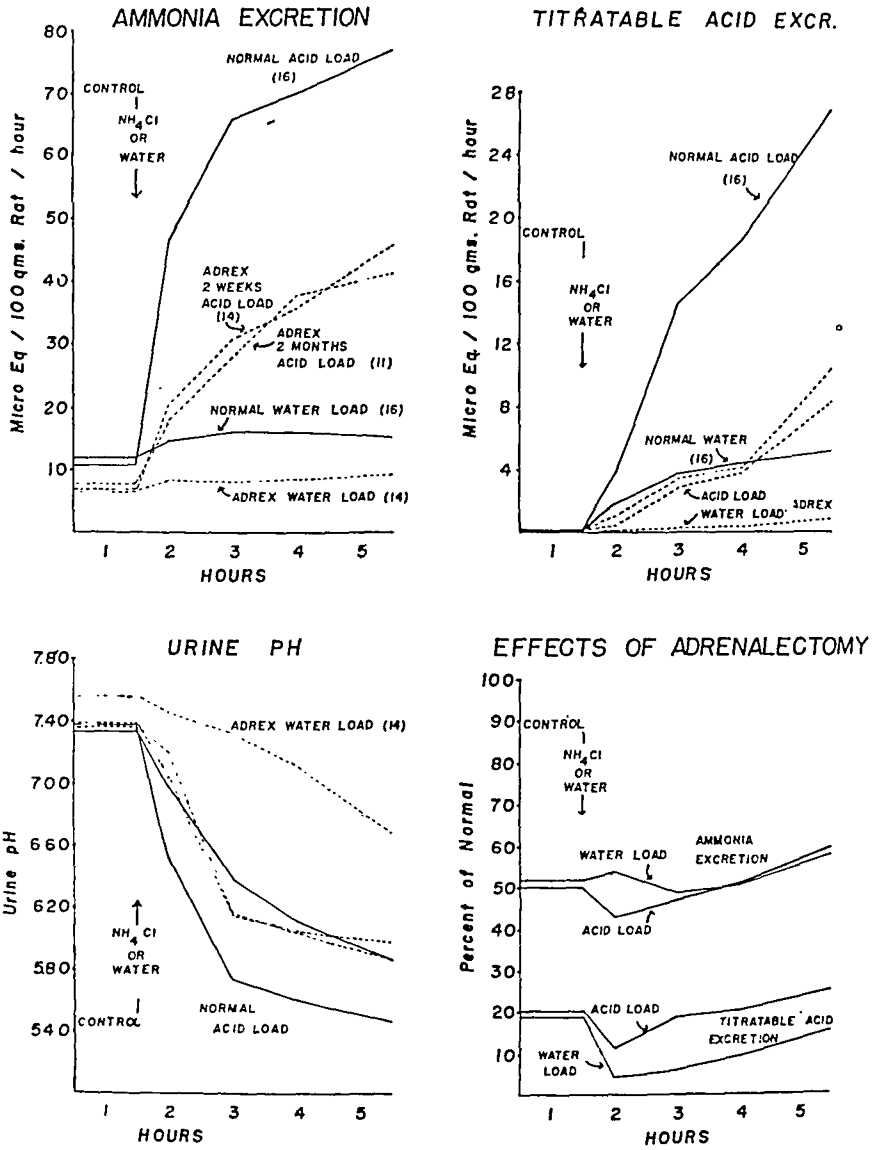

FIG. 1. The effects of adrenalectomy on the capacity of the acidotic rat to secrete ammonia and titratable acid.

In the lower right hand corner of Figure 1, the deficiency in capacity of the adrenalectomized rat to produce ammonia and titratable acid is made more apparent by plotting the output of these substances as a per cent of normal. It is quite evident that the adrenalectomized rat is capable of producing ammonia at a rate of only 50–60% of that of the normal animal, and that this deficiency remains relatively constant for both control and acidotic periods. The capacity of the adrenalectomized rat to form titratable acid is even more limited, amounting to but 15 to 25% of that of the normal animal.

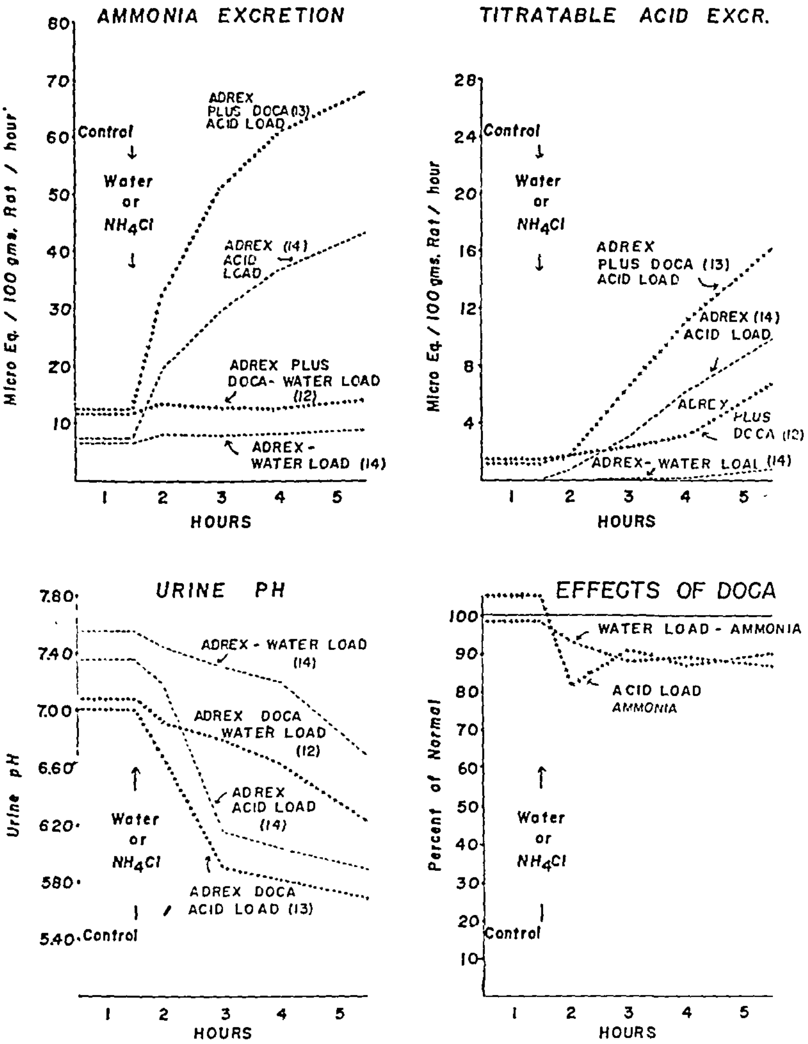

FIG. 2. The effect of desoxycorticosterone acetate on the capacity of the acidotic and adrenalectomized rat to secrete ammonia and titratable acid.

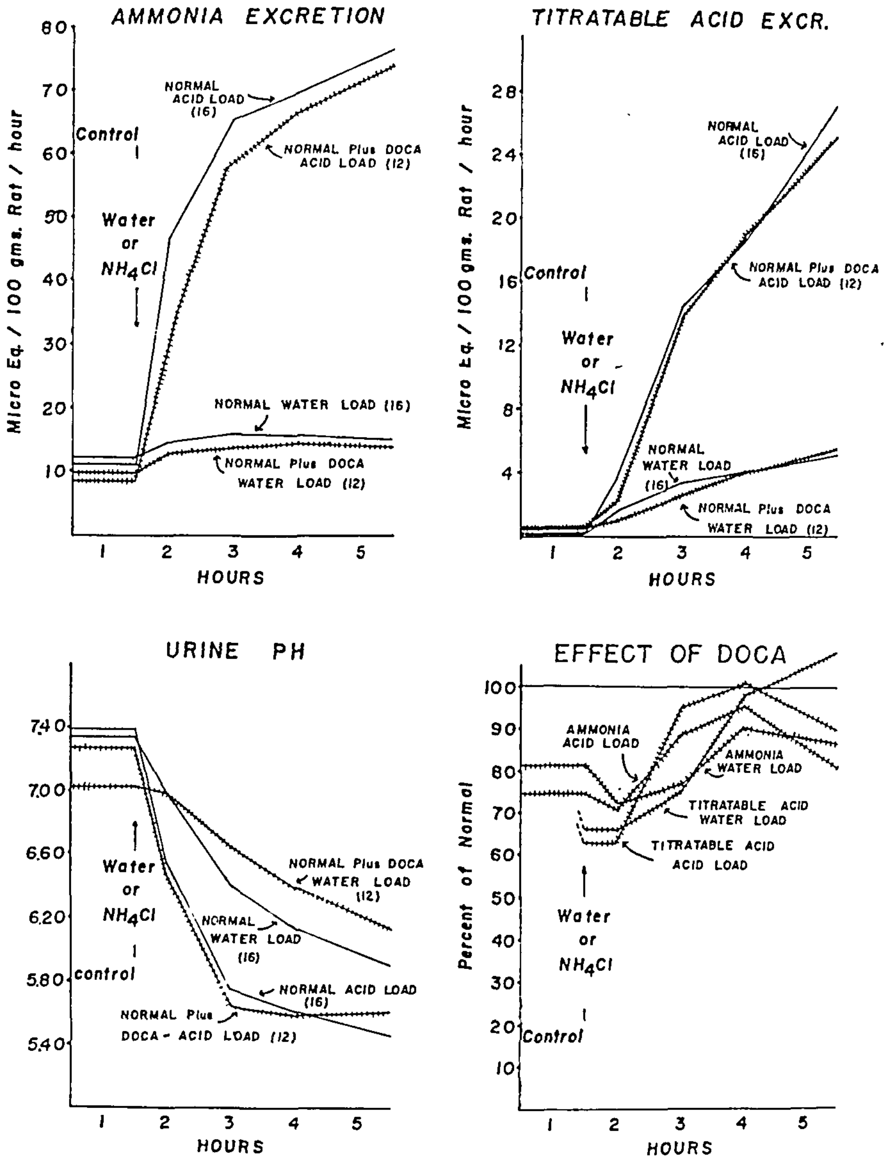

FIG. 3. The effects of desoxycorticosterone acetate on the capacity of the normal but acidotic rat to secrete ammonia and titratable acid.

Since an approximate quantitation of the reduced capacity of the adrenalectomized rat to secrete ammonium and hydrogen ions proved feasible by the administration of water or an acidifying agent in acute experiments, this technique was followed in assessing the activity of desoxycorticosterone acetate on the output of these ions species. Accordingly desoxycorticosterone was administered subcutaneously in dosage of 0.5 mg. per rat 18 hours prior to and again 1 hour prior to each experiment. The effects of the administration of his hormone to adrenalectomized rats are shown in Figure 2. It is apparent that desoxycorticosterone increased appreciably the renal output of ammonia and titratable acid, in the control periods, and in the presence of a mild or severe acidosis. The similarity of the excretory rates of these ions by the adrenalectomized rat receiving desoxycorticosterone with the rates of the normal rat as shown in Figure 1 is striking. The fact that there is almost complete restoration to the normal is seen in the lower portion of Figure 2 where it is apparent that the adrenalectomized rats receiving desoxycorticosterone have increased their capacity to secrete ammonia some 40% or to within 90 to 100% of the normal. Titratable acid is not represented on this chart since desoxycorticosterone increased the control rate of excretion several fold in comparison with the normal. However, the restoration of acid secretory function, though considerable, is not so complete as that of ammonia secretion.

With administration of desoxycorticosterone to the normal rat no increase in ammonia or titratable acid output is apparent. Indeed, almost identical excretory rates were obtained (Fig. 3). In two additional experiments on adrenalectomized and normal rats, adrenal cortical extract in dosage of 1 cc. per rat was administered subcutaneously 18 hours prior to and again 1 hour prior to the start of the experiment. No significant difference was found between the effects of adrenal cortical extract and desoxycorticosterone on ammonia and titratable acid excretion.

Inasmuch as 1) the adrenalectomized animal exhibited a greater capacity to secrete ammonia than hydrogen ions, and 2) desoxycorticosterone acetate or adrenal cortical extract was found capable of restoring these capacities to normal, it seemed profitable to investigate the temporal appearance of these deficiencies after acute bilateral adrenalectomy. Accordingly, experiments were performed in which all animals were either sham operated or adrenalectomized after the collection of two control periods. At the time of the operative procedure the animals were given 2 cc. of water per 100 grams body weight or ammonium chloride in dosage of 1 mg. per gram of body weight in a similar amount of water. In those experiments involving the administration of an acid load, a similar dose of ammonium chloride was administered 42 hours prior to, 24 hours prior to, and 18 hours prior to the start of the experiment. Following the operative procedure, urine was collected at 2 hour intervals for 12 successive collection periods.

It is apparent that ammonia and titratable acid excretory rates for both the sham operated and adrenalectomized animals rose sharply following the administration of an acid load (Fig. 4). The adrenalectomized rats, however, attained peak excretory rates at an earlier time and at lower values than did the intact rats. As was expected, the deficiency in capacity of the adrenalectomized rat to excrete titratable acid was of greater magnitude than the deficiency in capacity to excrete ammonia. With administration of a water load, similar deficienceis were noted.

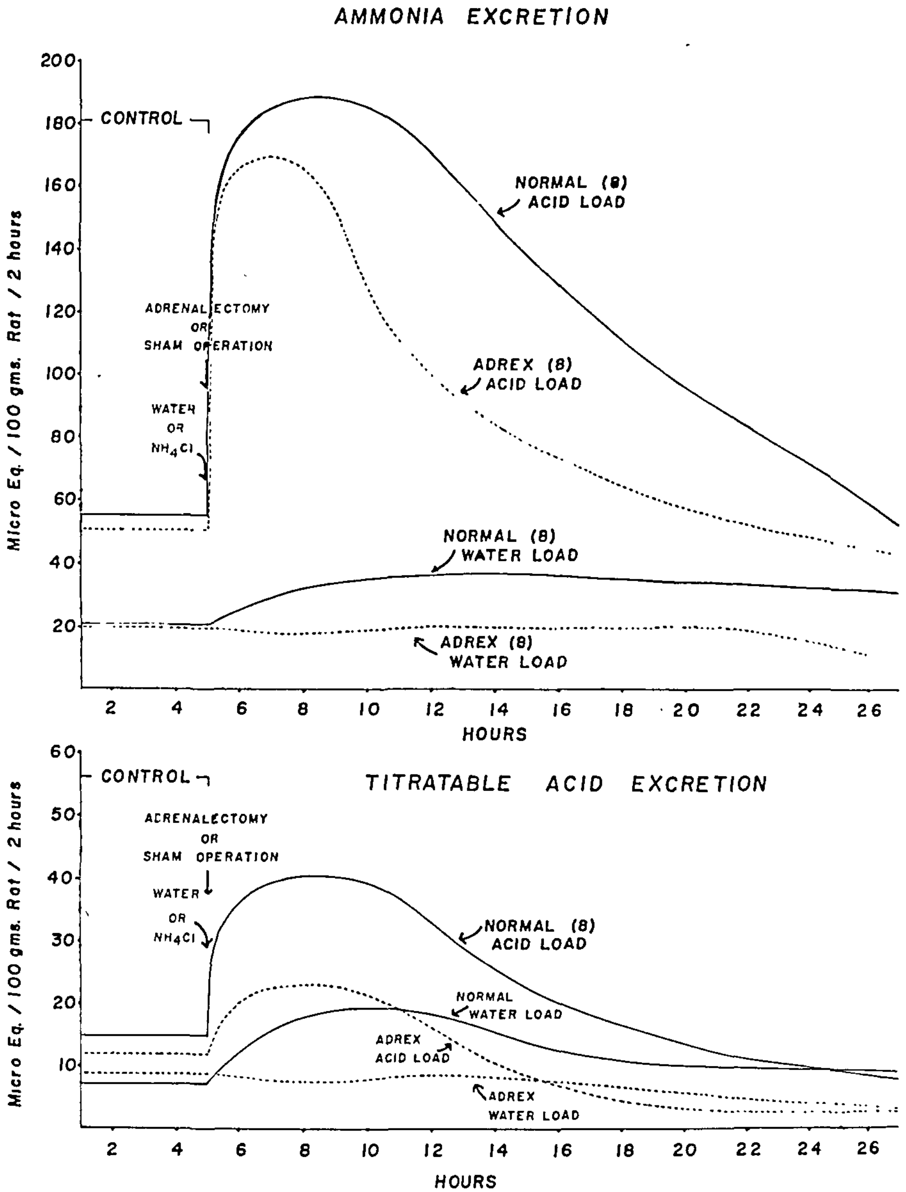

FIG. 4. The early time courses in the capacities of the sham-operated normal and acutely adrenalectomized rat to secrete ammonia and titratable acid.

It remained to be demonstrated that ammonium chloride actually stimulates the adrenal cortex. Evidence for stimulation of the adrenal cortex was obtained by administering ammonium chloride and then periodically assaying the adrenal cortices for their content of cholesterol and ascorbic acid. Urine collections were obtained at similar time intervals in order to compare the time of peak ammonia and titratable acid outputs with the time of minimum glandular cholesterol content.

As may be seen in Figure 5, the average cholesterol content fell from a control level of 0.778 mg. per 100 grams of body weight to a low of 0.514 mg. four hours after the administration of 1 mg. of ammonium chloride per gram of body weight. Peak ammonia and titratable acid excretion occurred six hours after the administration of the acid load. Ascorbic acid content of the adrenal gland fell from control values of 5.5 mg. per 100 grams body weight to a low of 4.54 mg. two hours after the acid loading and then gradually rose to somewhat elevated values.

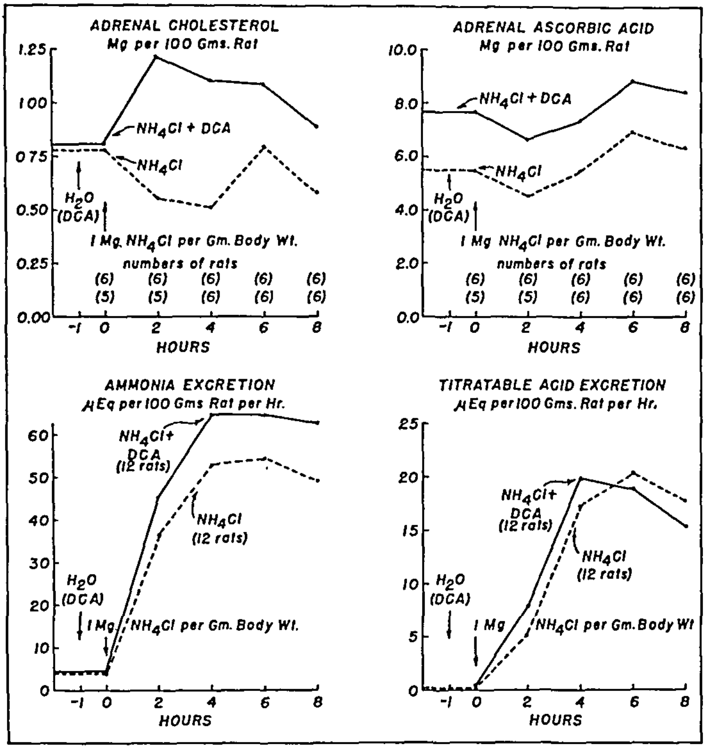

FIG. 5. The effects of ammonium chloride and ammonium chloride plus desoxycorticosterone acetate on the adrenal cholesterol and ascorbic acid content of the rat.

DISCUSSION

It is well established that the untreated adrenalectomized animal loses excessive quantities of sodium and chloride in the urine and in consequence suffers reduction in the plasma concentrations of these ions and in the volume of extracellular fluid as well (Loeb, 1932; Harrop *et al.*, 1933, 1936). However, an additional quantity of base is lost because of a relative deficiency in renal capacity to secrete hydrogen ions and ammonia (Jiminez-Diaz, 1936; Sartorius *et al.*, 1952). This deficiency accounts for the mild to moderate acidosis (reduction in bicarbonate bound base) of the ad-

COMPARISON OF POTENCY OF DIFFERENT ACTH PREPARATIONS BY INTRAVENOUS AND SUBCUTANEOUS ROUTES OF INJECTION

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MANY papers that indicate the existence of multiple ACTH factors have been published. Astwood (1952) and Reinhardt, Geschwind and Li (1951) have reviewed these papers recently. Most of the eivdence for two or more factors is based on quantitative differences in potency of preparations when assayed on the one hand by the ascorbic acid depletion method of Sayers, Sayers and Woodbury (1948) after intravenous injection (one hour test) and, on the other hand, by increase in adrenal weight or histological changes after subcutaneous injection (3–4 days).

Because of the differences in experimental method described in these reports, e.g., in route of injection, duration of treatment, and end point observed, the following experiments, in which only the route of injection was varied, were carried out. Hypophysectomized rats were injected subcutaneously or intravenously with two different types of preparations of ACTH. The left adrenal gland was removed one hour and the right adrenal two hours later for determination of the ascorbic acid content. It was found first, that a 100 to 1000 times larger dosage may be required subcutaneously than intravenously to cause a significant ascorbic acid depletion and second, that there was a large difference in the potency estimates with two different types of ACTH preparations depending upon the route of injection.

METHODS

Groups of male rats from Hormone Laboratories, Chicago, approximately 40 hours after hypophysectomy were injected either intravenously (nembutal anesthesia) or subcutaneously (no anesthesia) with 0.5 ml of aqueous solution of ACTH at 3 or more appropriate dosage levels, which cause a 20–50% ascorbic acid depletion. Under ether anesthesia, at one hour after injection, the left adrenal was removed, trimmed, weighed and placed in metaphosphoric acid solution. Two hours after injection the rats were killed with chloroform and the right adrenal removed and treated as was the left adrenal. Ascorbic acid content was determined by the method of Mindlin and Butler (1938). There were at least 3 rats at each dosage level.

ACTH PREPARATIONS

Frozen hog pituitary glands were ground and extracted twice at pH 2.8 using sulfuric acid and a solvent medium of 20% ethanol. The combined

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extracts were concentrated in vacuo to a volume of about 100 ml./lb. and an equal volume of acetone added. The precipitates that formed were removed and found to have little potency. The clear 50% acetone solution was run through an IR-120 column in the acid cycle. To the acid effluent from the column with a pH of about 1.6, two volumes of anhydrous acetone were added slowly, with stirring, making the acetone concentration about 84% by volume. The gummy protein precipitate which formed, was redissolved in a minimum volume of water and poured into 20 volumes of acetone. The *flocculent white precipitate of* ACTH *was removed* by decantation and centrifugation and thoroughly dried with acetone. Such preparations in general are soluble at all pH's in H_2O and have a potency of 2–6 I.U./mg. They are indicated as A preparations in Table 1 and Figures 1 and 2. P 327A had a higher potency because it had been refractionated in acid acetone.

TABLE 1. SHOWING THE RELATIVE POTENCY OF ACID, A, AND BASIC, B, ACTH PREPARATIONS WHEN ASSAYED BY TWO ROUTES OF INJECTION

(1) Preparation	(2) Dosage to cause 20% A. A. depletion		(3) Dosage ratio Subcut./I.V. from Col. (2)	(4) Ratio of A/B from Col. (3)
	I.V. µg.	Subcut. µg.		
E 3A	0.33	100	300	3
E 13B	0.33	33	100	
M 2160A	0.5	100	200	11.1
M 2161B	1.5	27	18	
P 327A	0.04	50	1250	10.4
D 13B	0.25	30	120	
C 12A	0.1	125	1250	6
C 15B	0.2	50	250	

The gland residue, after the second extraction above, was suspended in ethanol in an amount that resulted in an ethanol concentration of 65–70% by volume and NaOH added until the pH (glass electrode) was 9.5–10. The third and fourth extracts were made under these conditions, which were those used by Bates and Riddle (1935) to extract prolactin from bovine pituitaries. The extracts were neutralized to pH 6 and 2 volumes of ethanol added to precipitate the ACTH and other proteins. The precipitate was then extracted twice with 50% acetone at pH 9 and the extract adjusted to pH 3 where another precipitate was removed and re-extracted. The pH 3, 50% acetone soluble fraction was then deashed by use of an IR-120 resin column as for the first and second extracts but the effluent was made up to 90–95% acetone at once. The white precipitate was dried and contained the ACTH from the third and fourth or alkaline extracts.

Such preparations are insoluble in water between pH 3.5 and 6.5 and usually have a potency of 1 to 2 I.U./mg. They are indicated as B preparations in Table 1 and Figures 1 and 2.

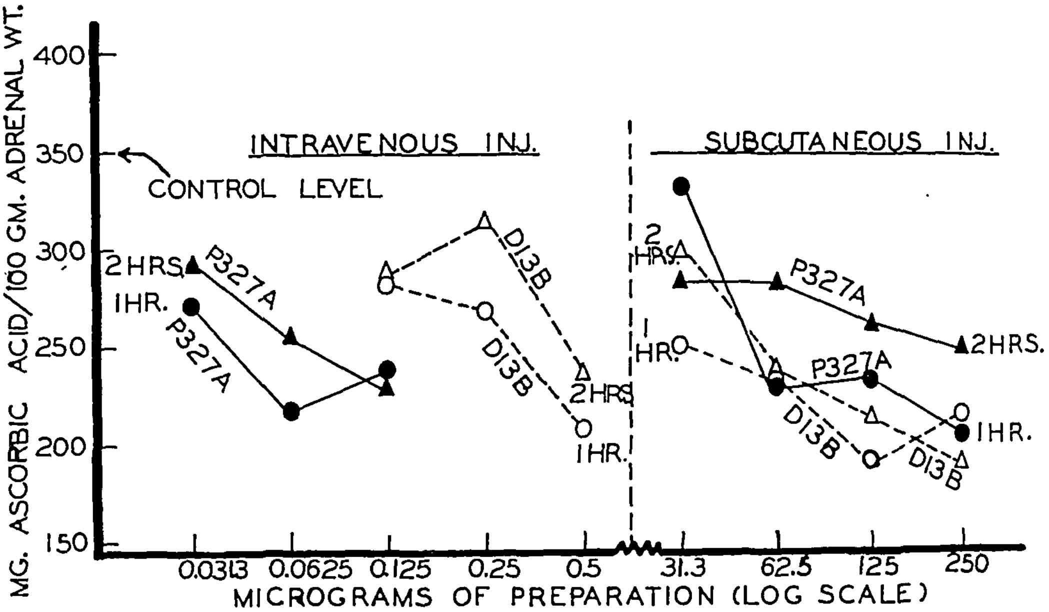

FIG. 1. Showing quantitative differences between two ACTH preparations injected by different routes.

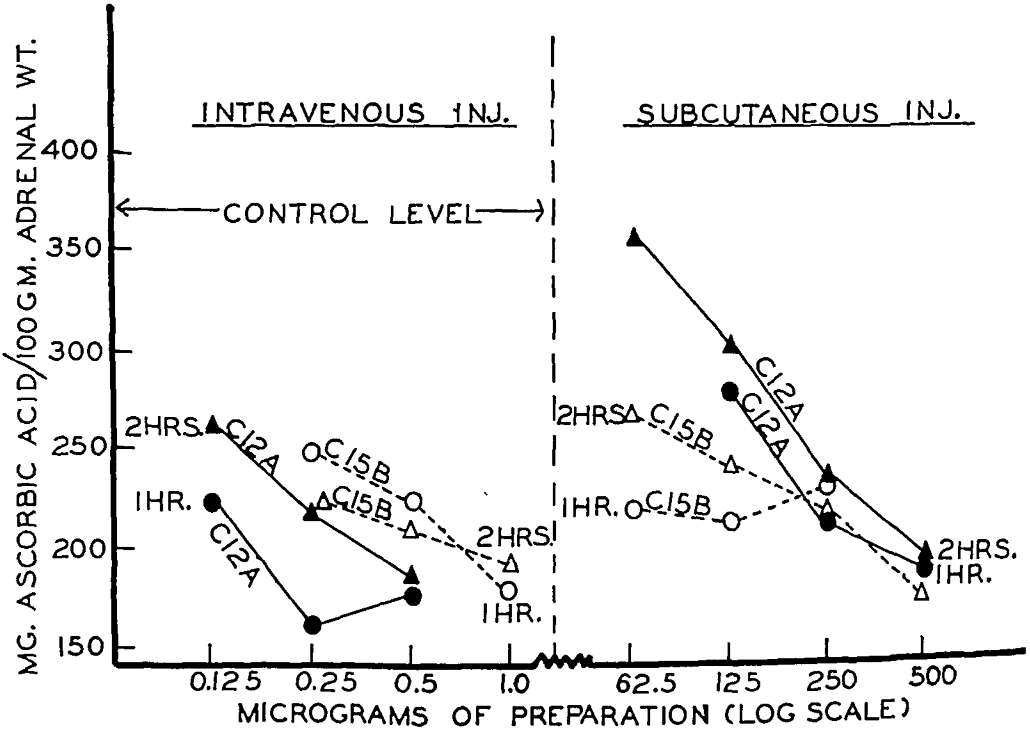

FIG. 2. Showing quantitative differences between two ACTH preparations injected by different routes.

RESULTS

Four comparisons were made between A extracts which were acid extracts and B extracts which were basic or alkaline extracts. A comparison of the effect of two pairs of these preparations upon the ascorbic acid levels in the adrenal when injected intravenously and subcutaneously are shown graphically in Figures 1–2. In both cases it can readily be seen that the A fractions are more potent than the B fractions by intravenous injection. In contrast, when given subcutaneously, the B preparations cause an equal or greater depletion of ascorbic acid than the A preparations.

Another highly significant fact shown in these figures is that the subcutaneous dosage required to cause 20–50% depletion of ascorbic acid is 100 or more times greater than the intravenous dosage. It is apparent from the figures that the slopes of the dosage response lines with different preparations are not always parallel especially when the subcutaneous route of injection is used.

In general, the ascorbic acid levels of the adrenals two hours after injection are slightly higher than after one hour indicating that the adrenal ascorbic acid level is beginning to return towards the control level. The ascorbic acid levels at the two time intervals are confirmatory of each other so that the comparisons may be considered as being carried out in duplicate. The two time intervals were used because we had previously found that the fall in ascorbic acid level after ACTH was injected intravenously was maximum in 10 to 15 minutes whereas it was not maximum until about one hour after ACTH was injected subcutaneously. It was thought desirable to consider time differences in the experiment due to the different rates of action of the ACTH by the different routes of injection.

By averaging the one and 2 hour data and by graphical interpolation and extrapolation, an estimate was made of the dose to cause a 20% ascorbic acid depletion by the different routes of injection. This is shown in column 2 of Table 1. These estimates, of course, are subject to considerable error due to the small number of animals used, the variability of the amount of depletion in different rats and the differences in slope, especially the tendency for horizontal slope with the subcutaneous route. In our laboratories 0.5 to 1 milliunit of the International Standard, when given intravenously, will cause a 20% depletion of the ascorbic acid level. Thus, preparation P 327A has a potency of 10 to 20 I.U./mg., and D 13B of 2 to 4 I.U./mg. In column 3 it is shown in the case of P 327A that 1250 times more ACTH is required subcutaneously than intravenously to cause a 20% ascorbic acid depletion whereas with D 13B only 120 times as much ACTH is required.

Column 4 shows how much the relative potency of the A and B fractions differ with the different routes of injection. Thus E 3A would be only $\frac{1}{3}$ as potent as E 13B if the potency were determined by the subcutaneous route whereas they are of equal potency by the intravenous route. Likewise P 327A, which is approximately 6 times more potent than D 13B by intravenous assay is only 60% as potent as D 13B by subcutaneous assay making an over-all difference of potency of 10.4 times depending upon whether the intravenous or subcutaneous estimates of potency were used.

An additional experiment was run comparing intraperitoneal and subcutaneous routes of injection. The dosage ratios by the two routes expressed as in Table I, column 3, (Subcut./I.P.) were 1.1 for M 2160A and 1.33 for M 2161B, which values are not significantly different from 1.0.

A preliminary experiment with subcutaneous injection twice daily for four days of P 327A and D 13B into hypophysectomized rats indicated that they were about equal in adrenal weight increasing potency. A 50% increase in adrenal weight was obtained with 6.7 mg. and 3.3 mg. respectively. As would be expected, the adrenal weight increasing potency was more nearly correlated with the ascorbic acid depletion tests when injections were made subcutaneously than when made intravenously.

Unpublished results, using adrenal ascorbic acid depletion in normal rats as the end point, upon duration of action of single subcutaneous injections of 16 I.U. of ACTH formulated in sesame oil in a manner to slow absorption (see also Astwood, 1952), show that ACTH preparations like A and B can be made to increase adrenal weight by over 50% in 24 hours and 100% in 48 hours while maintaining ascorbic acid levels more than 50% below control levels. This remarkable increase in adrenal weight is due to the slow release of ACTH over a period of days, as shown by the ascorbic acid levels, and not to any special qualitative property of the ACTH in the preparations. Numerous clinical reports of the high potency of ACTH given by slow intravenous infusion have been made (Mandel, Singer, Gudmundson, Meister and Modern, 1951; Renold, Forsham, Maisterrana and Thorn, 1951).

DISCUSSION

Astwood and coworkers (1952) have made preparations of ACTH by adsorption on oxycellulose that are 25 to 100 times more potent than the ones used here. Hence, the preparations used here and those used by most other investigators contain 95% or more of non-ACTH protein or protein-like material. The different ascorbic acid depletion potencies of the A and B preparations, shown above, and different solubilities probably are not due to the ACTH itself but are due to differences in the 95% contaminants and their effect upon the rate of absorption from the site of injection.

Different estimates of potency by the different routes of administration are important clinically because most ACTH is used by routes of injection other than the intravenous route which is used for standardization. Assay of ACTH preparations by the universally used intravenous Sayers or Munson methods probably does not properly indicate the clinical efficacy of the preparation when given intramuscularly. Conversely a subcutaneous assay method would not properly indicate the intravenous efficacy of a preparation. In all 4 cases in Table 1 the iso-electrically precipitable fraction, B, is much more potent subcutaneously than the intravenous assay would indicate it to be. Clinical proof that a similar difference between these preparations occurs in man is not available.

Most differences in physiological efficacy of ACTH are as readily explained on the basis of different rates of absorption, which depends upon the route of injection and the nature and formulation of the preparation, as on the basis of a qualitative difference in the ACTH present, i.e., the multiple factor theory.

SUMMARY

The potencies of two different types of ACTH preparations were compared by intravenous and subcutaneous injection using adrenal ascorbic acid depletion in the hypophysectomized rat as the criteria of potency. Estimates of the relative potency of the two types differed with the route of injection by 3 to 11 times. The subcutaneous route of injection required as much as 100 to 1000 times larger dosage than did the intravenous route to cause equivalent ascorbic acid depletion. The data given are interpreted as not supporting the multiple ACTH factor therory.

Acknowledgements

This work was done with the cooperation of the Squibb Biological Control Laboratories under Dr. Roger Varney.

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RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM AND SIZE OF NITROGEN POOL DURING NITROGEN STORAGE INDUCED WITH TESTOSTERONE PROPIONATE, AND TESTOSTERONE PROPIONATE COMBINED WITH GROWTH HORMONE

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IN THE human, normal growth appears to occur in two phases: prepuberal growth, hypothesized as being entirely referable to pituitary growth hormone, and puberal growth referable to steroid growth promoting factors from the gonads and the adrenal cortex (Kinsell *et al.*, 1948). Nitrogen retention and protein anabolic effects of testosterone and its derivatives, methyltestosterone and particularly testosterone propionate have received considerable attention, and, although much factual information has been obtained (Kochakian, 1946, 1950), elucidation of the mechanism of these effects is still awaited.

Technical procedures and mathematical treatment developed by Sprinson and Rittenberg (1949) and Hoberman (1949–50) for assessing protein and amino acid metabolism in terms of rates of protein synthesis, amino acid catabolism, and size of the nitrogen pool have been utilized in characterizing and quantitating the over-all mechanisms involved in nitrogen storage induced in the adult female dog with anterior pituitary growth hormone (Bartlett and Gaebler, 1952). The effect of growth hormone in depressing the rate of amino acid catabolism and increasing the size of the nitrogen pool was particularly striking and as previously suggested (Bartlett and Glynn, 1950) perhaps indicative of the mechanism by which growth hormone regulates the intracellular synthesis of protein.

Extension of these studies to include nitrogen storage induced in the adult female dog with testosterone propionate seemed particularly desirable not only from the standpoint of comparing the pattern of the over-all mechanisms involved in the metabolism of proteins and amino acids with the pattern observed during nitrogen storage induced by growth hormone but also because of widespread clinical interest in the use of androgens to promote nitrogen retention. It was felt that information of this sort might also be of value in extending our knowledge and understanding of the role of androgens in the regulation of puberal growth. In

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view of the summation effect of testosterone propionate and growth hormone on body weight and protein anabolism of castrated mice (Kochakian and Stettner, 1948), it also seemed of interest to include experiments in which nitrogen storage was induced by the simultaneous administration of growth hormone and testosterone propionate at dose levels corresponding to those given in single experiments.

EXPERIMENTAL

Animals—Dogs 44, 52, and 55 utilized in the preceding study with growth hormone (Bartlett and Gaebler, 1952) and maintained in the manner previously described (Gaebler, Bartlett and Sweeney, 1951) were used in the experiments reported herein.

Procedure—Since data were available on the rates of protein synthesis, amino acid catabolism, and size of the nitrogen pool for each of these animals during nitrogen balance experiments (Bartlett and Gaebler, 1952), it was only necessary to conduct experiments in which nitrogen storage was induced with testosterone propionate or the combination of testosterone propionate with growth hormone. The experimental procedure followed and the mathematical treatment of the data were essentially the same as previously described. Constancy of both body weight and total urinary nitrogen excretion were taken as indicative of nitrogen equilibrium and nitrogen storage was induced in such animals either by the intramuscular injection of testosterone propionate at dose levels of 50 mg./day for 5 days, or by a combination of this level of androgen with the subcutaneous administration of growth hormone in doses of 5 mg./day for 5 days. N^{15}-labeled glycine was administered on the 4th day of induced nitrogen storage experiments and urinary excretion of the N^{15} label determined at 24 and 48 hours after ingestion of the amino acid.

RESULTS

In Table 1 are summarized the results of experiments in which nitrogen storage was induced with testosterone propionate. The rate of protein synthesis, S_k, and the size of the nitrogen pool, P_k, were calculated according to the mathematical treatment of Sprinson and Rittenberg (1949), and, k_1 and k_2, rates of amino acid catabolism and incorporation of amino acids into protein, by the mathematical treatment of Hoberman (1949–50). S_k values previously reported (Bartlett and Gaebler, 1952) for Dogs 44, 52, and 55 during nitrogen balance experiments were respectively in contrast to those given in Table 1—0.49, 0.56, and 0.82 gm. N/kg. body wt./24 hrs., and P_k values were respectively 0.82, 0.91, and 0.95 gm. N/kg. body wt. Values of k_1 abstracted from the same reference were respectively 3.85, 3.96, and 2.74%/hr. and values for k_2 3.85, 4.19, and 3.60%/hr. In general, the rate of protein synthesis was higher and the rate of amino acid catabolism lower during nitrogen storage induced with testosterone propionate than during the nitrogen balance experiment. There appeared to be no significant change in the size of the nitrogen pool, P_k.

Results of experiments in which nitrogen storage was induced in Dogs 44 and 55 by the combination androgen and growth hormone treatment are presented in Table 2. Comparison of this data with the nitrogen balance

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM AND SIZE OF THE NITROGEN POOL

Experimental dog[1]	Wt. gain[2] (kg.)	N stored[3] (gm.)	Rate of protein synthesis, S_k (gm. N/kg. body wt./24 hr.)	Size of N pool, P_k (gm. N/kg. body wt.)	Fraction of amino acid N	
					Catabolized and excreted, k_1 (%/hr.)	Incorporated into protein k_2 (%/hr.)
44	0.31	3.89	0.65	0.82	2.18	4.70
52	0.14	2.66	0.79	0.81	3.19	5.51
55	0.43	3.40	0.66	1.05	2.38	3.52

[1] Nitrogen storage induced in Dogs 44, 52 and 55 with Schering's Oreton brand of testosterone propionate.

[2] Weight gain was computed by determining the difference between the maximum weight attained during the 5 day period of induced nitrogen storage and the weight of the animal preceding induction of nitrogen storage.

[3] Nitrogen storage was calculated from total urinary nitrogen excretion alone. The average of 5 or more days preceding induction of nitrogen storage was taken as the control level and the difference between this value and the urinary nitrogen excretion for each day of the 5 day period determined. The values given in the table are summation values for the 5 day period of storage.

data from the same animals results in a number of interesting observations. In spite of the induction of marked nitrogen storage and gain in body weight as a result of the combined treatment with androgen and growth hormone, the rate of protein synthesis calculated in terms of either S_k or k_2 was not appreciably affected. Rates of amino acid catabolism were decreased in both animals and the size of the nitrogen pool was reduced.

TABLE 2. RATES OF PROTEIN SYNTHESIS, AMINO ACID CATABOLISM, AND SIZE OF THE NITROGEN POOL DURING NITROGEN STORAGE INDUCED WITH TESTOSTERONE PROPIONATE PLUS GROWTH HORMONE

Experimental dog[1]	Wt. gain[2] (kg.)	N stored[3] (gm.)	Rate of protein synthesis, S_k (gm. N/kg. body wt./24 hr.)	Size of N pool, P_k (gm. N/kg. body wt.)	Fraction of amino acid N	
					Catabolized and excreted, k_1 (%/hr.)	Incorporated into protein, k_2 (%/hr.)
44	0.71	10.88	0.47	0.58	1.86	3.94
55	0.79	11.18	0.74	0.87	2.28	4.30

[1] Nitrogen storage was induced in Dog 44 with testosterone propionate (Oreton) combined with growth hormone preparation Rx099916, supplied through the courtesy of Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis & Company, and, in Dog 55 with testosterone propionate (Oreton) combined with growth hormone preparation 22KR1 supplied by Armour and Company.

[2,3] Please refer to footnote of Table 1.

DISCUSSION

The observation of a decrease in the rate of amino acid catabolism, k_1, in the normal adult female dog during nitrogen storage induced with testosterone propionate is in agreement with the observation that decreased urinary urea excretion in this animal is not due to elevated blood nonprotein nitrogen (Gaebler and Tarnowski, 1943). In terms of rates of protein synthesis and amino acid catabolism, nitrogen storage induced in the adult female dog with testosterone propionate appears to be qualitatively similar to nitrogen storage induced with growth hormone (Bartlett and Gaebler, 1952), *i.e.*, characterized by a higher rate of protein synthesis and a lower rate of amino acid catabolism than observed in the same animal during nitrogen balance. Testosterone propionate in contrast to growth hormone appears to have no significant effect on the size of the nitrogen pool.

In considering changes in the rates of the principal over-all reactions required to either increase or decrease the size of the metabolic pool of nitrogen, relative to nitrogen balance values, a decreased rate of protein synthesis or amino acid catabolism or an increased rate of protein degradation might singly or in one of several combinations result in a net increase in the size of the nitrogen pool, while an increased rate of protein synthesis or amino acid catabolism or a decreased rate of protein degradation might similarly result in a net decrease in the size of the nitrogen pool. In view of the fact that the pattern of protein metabolism in terms of rates of protein synthesis and amino acid catabolism is qualitatively similar in nitrogen storage induced by either growth hormone or testosterone propionate, the contrast in the net effect on the size of the nitrogen pool seems of special interest. The complexity of the situation and the difficulty involved in its interpretation is emphasized with the realization that the net effect on the size of the nitrogen pool could, in addition to those factors previously noted, also be determined by either the extent to which the rate of protein synthesis was increased and the rate of amino acid catabolism decreased or by changes in the rate of protein degradation or by a combination of these contributing factors. The importance and necessity of having data on the relative rates of protein degradation during both nitrogen balance and nitrogen storage experiments seems quite apparent, and until such data are available, progress in the elucidation of the mechanisms involved in nitrogen storage and growth and particularly in respect to the accumulation of protein will be limited.

The observation that growth hormone (Bartlett and Gaebler, 1952) and testosterone propionate administered alone increased the rate of protein synthesis, but when administered in combination had no significant effect on this value, and the observation that the rate of amino acid catabolism was decreased are of special interest. The fact that the size of the nitrogen pool actually decreased under these conditions suggests that the combination androgen and growth hormone treatment resulted in a significant reduction in the rate of protein degradation.

In connection with the hypothesized endocrine regulation of prepuberal and puberal growth (Kinsell *et al.*, 1948), it should be noted that growth during the puberal period, which is particularly characterized by the accumulation of body protein (*e.g.*, in the development of the musculature), might also be referable to a combination of growth hormone and steroid growth promoting factors such as the androgens and estrogens. If such is the case, and, the results of our experiments in which nitrogen storage was induced in the adult female dog with growth hormone and in combination with testosterone propionate can be interpreted respectively in terms of simulated prepuberal and puberal growth then the latter in contrast to prepuberal growth may be largely due to a decrease in the rate of protein degradation. The relatively high protein requirements and output of nitrogen during the phase of prepuberal growth as compared with that during puberal growth might be taken as evidence supporting the view that prepuberal growth is characterized by a high level of protein metabolism, *i..e.*, a high rate of protein synthesis and a high rate of protein degradation, whereas, puberal growth is characterized by a relatively lower level of protein metabolism, *i.e.*, relatively lower rates of protein synthesis and protein degradation.

SUMMARY

Nitrogen storage induced in the adult female dog with testosterone propionate is characterized by an elevation in the rate of protein synthesis and a decrease in the rate of amino acid catabolism relative to values observed in the same animal during nitrogen balance experiments.

Testosterone propionate had no significant effect on the size of the metabolic pool of nitrogen in the adult female dog.

A combination of growth hormone and testosterone propionate produced marked nitrogen storage and gain in body weight, a decrease in the rate of amino acid catabolism and the size of the nitrogen pool in the adult female dog. The rate of protein synthesis was unaffected in the same animal.

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THE INTERCONVERSION OF ESTRONE AND ESTRADIOL-17β BY RAT LIVER SLICES[1,2,3]

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SINCE Zondek's original demonstration (1934) that rat liver mince "inactivates" estrogens, many workers have engaged in the study of the *in vitro* metabolism of these steroids employing loss of biological activity as a means of following hormone degradation (Heller, 1940; Engel, P. and Rosenberg, 1945; Twombly and Taylor, 1942; Levy, 1947 and DeMeio *et al.*, 1948). Pearlman and DeMeio (1949) accomplished the only isolation of estrone after the incubation of estradiol-17β[5] with rat liver slices. A technique for the isolation and determination of estrogens in minute quantities would be of great assistance in studying this reaction in detail. Counter-current distribution and fluorometric analysis have provided a means for the separation, quantitative estimation and identification of natural estrogens in urinary extracts (Engel *et al.*, 1950). This report describes the application of these techniques to the analysis of estrogens after incubation with rat liver slices.

METHODS

Tissue Preparation: Female Wistar strain rats, 6–10 months old were decapitated and bled. Their livers were quickly removed, chilled, sliced to 0.5 mm. thickness with a Stadie-Riggs microtome, drained and weighed. Cirrhosis and hepatomas were induced in similar rats by the administration of a high fat diet containing p-dimethylaminoazobenzene (Kline *et al.*, 1946) and prepared for incubation as described above. When hepatomas were used for incubation, they were dissected free from surrounding tissue. Microscopic sections were prepared and examined to insure purity of the preparation.

Hormone Preparation: Estradiol was analyzed by the counter-current, fluorometric

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[2] A preliminary report of this work was presented at the Second International Congress of Biochemistry, Paris, France, July 21–26, 1952.

[3] This is publication #777 of the Cancer Commission of Harvard University.

[4] This paper is abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors in Biological Chemistry.

[5] Hereafter referred to as estradiol. Nomenclature employed is that of Fieser and Fieser (1949).

and estradiol were incubated with boiled liver and an average recovery of 76% was obtained (Fig. 2). Owing to changes in the physical state of the tissue proteins, the extraction of estrogens from boiled liver is rendered more difficult.

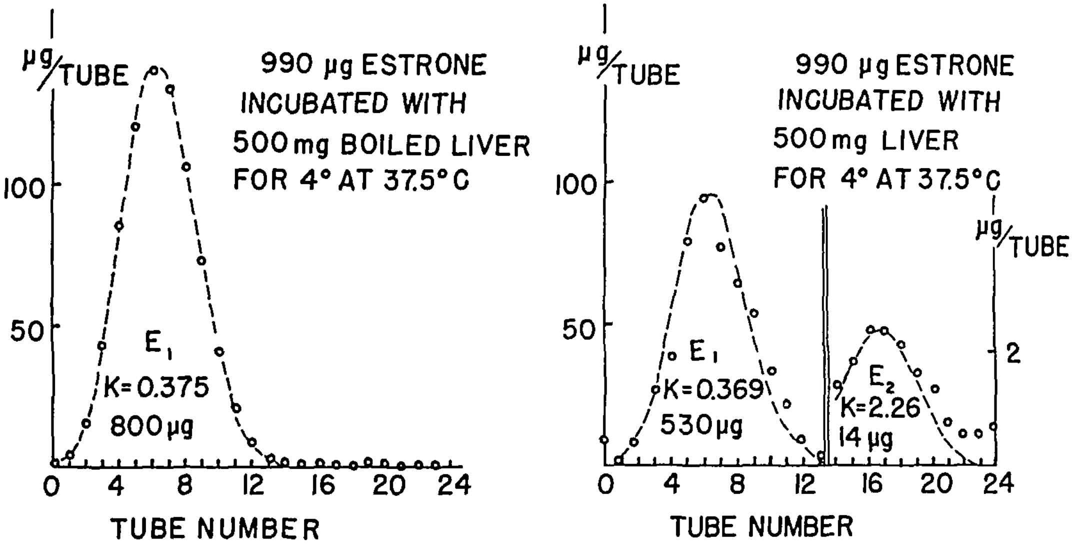

FIG. 2. Incubation of estrone with rat liver slices. Typical counter-current distribution curves. Intact liver slices converted estrone to estradiol. Boiling the tissue prevented conversion. E_1—Estrone; E_2—Estradiol; K—Partition Coefficient; ₒ—Experimentally determined points; - - - —Theoretical.

TABLE 1. EFFECT OF VARYING THE HORMONE SOLVENT ON THE AEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY NORMAL RAT LIVER SLICES

Solvent	Initial estradiol	Total recovery	Estradiol recovered		Estrone yield		
	µg	%	µg	K*	µg	%	K*
Aqueous	970	46%	430	2.1	18.5	1.9	0.34
	970	63	605	2.2	9.2	0.9†	0.31
	970	29	276	2.0	7.5	0.8	0.36
	490	64	294	2.1	18.2	3.7	0.39
	900	43	365	2.1	22.0	2.5	0.38
Ethanol	1020	64	555	2.1	93.0	9.1	0.33
	1020	26	222	2.1	39.8	3.9	0.36
	1020	22	188	2.3	36.1	3.5†	0.36
	1020	48	440	2.0	47.0	4.6	0.36
	1020	68	633	2.2	61.2	6.0‡	0.35
Aqueous with added Ethanol	980	37	336	2.0	21.4	2.2	0.39
	660	46	260	2.1	38.3	5.8	0.41
	750	35	233	2.1	31.0	4.2	0.38

* K—Partition coefficient in 50% MeOH—50% H_2O/CCl_4
†—2 hour incubation.
‡—Intracellular buffer.

Incubation of Estradiol with Normal Liver Slices (*Table 1*): When estradiol in ethanol solution was incubated with normal rat liver slices using a hormone concentration of 0.02 mg./mg. tissue/ml. buffer, there was an average yield of 5.4% estrone and a recovery of 40% of unchanged estradiol. This is in close agreement with the data of Pearlman and DeMeio (1949) who isolated 6% estrone and recovered 40% estradiol after incubation of the hemisuccinate of estradiol with rat liver slices. The 55% of starting material unaccounted for represents conversion of estrone and/or estradiol to unknown metabolites.

TABLE 2. EFFECT OF VARYING THE HORMONE CONCENTRATION ON THE AEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY NORMAL RAT LIVER SLICES

Hormone concentration*	Initial estradiol µg	Total recovery %	Estradiol recovered		Estrone yield		
			µg	K	µg	%	K
0.1	5100	72	3630	2.2	51	1.0	0.35
	1020	45	450	2.1	14	1.4	0.37
0.02 (Aver. of 3 runs Table I)	1020	46	408		54	5.9	
0.006	1020	33	191	2.1	147	14	0.37
	1020	0.3	1.8	2.1	1.2	0.1	0.37

*—Hormone concentration = mg. hormone/mg. liver/ml. buffer.

An experiment employing an intracellular buffer is listed in Table 1 but the results are not strikingly different from those obtained with the conventional Krebs' solution. Results similar to these have been reported by DeMeio *et al.* (1948).

In the experiments cited above, alcohol was used as solvent to disperse the estradiol in the buffer solution. The amount of ethanol used was similar to that employed by DeMeio (1948). To test the possible effect of the ethanol on the metabolic reaction, the sodium salt of estradiol was prepared and added to the buffer solution. When the pH was adjusted to 7.4 the hormone was precipitated as a fine dispersion. The results of using this aqueous dispersion are shown in Table 1. When an 0.02 hormone concentration was used, the average yield of estrone was 2.7% and the recovery of estradiol was 49%. If the incubations were carried out with aqueous dispersions of the steroids (prepared from the sodium salts) to which ethanol was added, the yield of estrone was increased to 4% and the recovery of estradiol was 39%. The amount of ethanol was equal to the quantity used in the experiments in which this solvent alone was employed.

When the hormone concentration was increased to 0.1, the yield of estrone fell to 1%. Changing the hormone concentration to 0.006 increased both the destruction of estradiol and the yield of estrone (Table 2). In one experiment of this series, less than 1% of the steroid was recovered (cf. Pearlman and DeMeio, 1949). These data on the effect of changing hormone concentration are based on a small number of experiments in which there is a great deal of variation; nevertheless, a trend is apparent.

TABLE 3. AEROBIC CONVERSION OF ESTRADIOL* TO ESTRONE BY DISEASED RAT LIVER

Pathology	Hormone concentration	Total recovery %	Estradiol recovered		Estrone yield		
			μg	K	μg	%	K
Cirrhosis	0.02	74	735	2.0	13.1	1.3	0.33
	0.02	64	605	2.0	40.5	4.0	0.35
Hepatoma	0.02	80	805	2.1	5.9	0.6	0.33
	0.02	41	407	2.0	10.3	1.0	0.31
	0.02	70	710	2.3	6.8	0.7	0.35
Cirrhosis	0.006	60	470	1.8	138	14	0.33
	0.006	16	106	2.1	60	5.9	0.36
	0.006	1.5	0.8	2.0	0.7	0.07	0.42
Hepatoma	0.006	7.0	4.0	2.2	3.0	0.3	0.34

*—Initial estradiol = 1 mg.

TABLE 4. ANAEROBIC CONVERSION OF ESTRADIOL TO ESTRONE BY RAT LIVER SLICES

Solvent	Initial estradiol μg	Total recovery %	Estradiol recovered		Estrone yield		
			μg	K	μg	%	K
Aqueous	490	96	440	2.1	31.9	6.5	0.36
Ethanol	540	72	376	2.1	13.2	2.5	0.38

Incubation of Estradiol with Cirrhotic Livers and Hepatoma Nodules (Table 3): When estradiol was incubated with slices of cirrhotic liver or with hepatoma nodules using a hormone concentration of 0.02 mg. hormone/mg. liver/ml. buffer the yield of estrone was much lower and the recovery of estradiol was somewhat higher than with normal tissues. When the hormone concentration was decreased to 0.006, the difference between normal, cirrhotic and malignant tissues become less marked. The variations observed in the cirrhotic livers may be attributed to differences in the severity of the disease.

Anaerobic Experiments (Table 4): When estradiol in aqueous solution was incubated in evacuated Thunberg flasks, there was some destruction of the estradiol and conversion to estrone.

TABLE 5. CONVERSION OF ESTRONE TO ESTRADIOL BY NORMAL RAT LIVER SLICES

Solvent	Gas phase	Initial estrone µg	Total recovery %	Estrone recovered		Estradiol yield		
				µg	K	µg	%	K
Ethanol	air	990	60	585	0.37	14.1	1.4	2.1
	air	990	55	530	0.37	13.8	1.4	2.3
Aqueous	air	900	45	620	0.38	2.0	0.2	2.1
Aqueous with added Ethanol	air	950	52	480	0.38	10.0	1.1	2.1
Ethanol	N_2	495	82	380	0.35	24.5	5.0	2.2

Incubation of Estrone: When estrone was incubated with rat liver slices in air or nitrogen, conversion to estradiol occurred by the yield depended on whether an aqueous or ethanol solution was used (Table 5). An experiment employing an aqueous solution of estrone with added ethanol gave results similar to those found with alcohol alone. These findings are similar to those obtained with estradiol under these varying conditions (Table 1).

DISCUSSION

Methods: In an effort to obtain quantitative data and study subtle changes, counter-current distribution and fluorometry have been employed in the present work. It has been possible by these methods to characterize as little as 1 microgram of estrogen as either estradiol or estrone and to reproduce partition coefficient within standard diviations of ±0.02 for estrone and ±0.1 for estradiol for hormones extracted from incubation mixtures (Table 6). From the distribution data, it is possible to calculate the amounts and purities of the components present and in addition characterize them by their partition coefficients (Engel, Slaunwhite, Carter and Nathanson, 1950).

Interconversion of Estradiol and Estrone: The data presented in Tables 1 and 5 demonstrate the interconversion of estradiol and estrone. Under the

TABLE 6. REPRODUCIBILITY OF PARTITION COEFFICIENTS BY COUNTER-CURRENT DISTRIBUTION—24 TRANSFERS IN 50% $MeOH/CCl_4$

Compound recovered	Number of distributions	Partition coefficient	Standard deviation	Extracted from	Reference
Estrone	85	0.36	±0.02	Incubated tissue	Present study
	29	0.34	±0.02	Urine	(Slaunwhite)
	—	0.33	—	Pure compound	(Engel, 1950)
Estradiol	80	2.1	±0.1	Incubated tissue	Present study
	22	2.1	±0.1	Urine	(Slaunwhite)
	—	2.1	—	Pure compound	(Engel, 1950)

conditions employed, the reaction appears to favor the accumulation of the later compound. The nature of the solvent and the relative amount of substrate significantly affect this reaction (Tables 1 and 2).

The relative insolubility of estrone and estradiol in aqueous media has necessitated using organic solvents to disperse the hormones in the incubation mixture. It is possible that ethanol has an inhibitory effect on oxidative enzymatic reactions. Such an inhibition seems borne out by the comparison of results of incubation of estradiol and estrone with and without ethanol. Alcohol may inhibit the oxidative breakdown of estrone, allowing its accumulation without affecting the estrone-estradiol interconversion. The latter reaction is probably catalyzed by a dehydrogenase system (DeMeio *et al.*, 1948; Coppedge *et al.*, 1948), which is unaffected by ethanol in the concentrations used. Comparable total recoveries in the aqueous and alcohol experiments make solubility differences between the two solvents less likely as a factor.

The hormone concentration also appears to be a critical factor not only in the interconversion of estradiol and estrone, but in the further changes of these compounds. The apparent discrepancies in extents of estrogen "inactivation" (Heller, 1944) which have been reported by investigators using different hormone concentrations may be explained by the dependence of the reactions on hormone concentration. If a dehydrogenase system is involved in estrogen metabolism as has been suggested by DeMeio *et al.* (1948) and Coppedge *et al.* (1948) then the critical factor related to the amount of tissue present may be the associated concentration of diphosphopyridine nucleotide. This hypothesis is supported by the differential increase of estrone yield over estradiol recovery as the hormone concentration is decreased. In other words, as the hormone concentration is decreased by increasing the amount of tissue present, there is a differential increase of dehydrogenase over oxidative activity.

Although the conversion of estrone to estradiol has been demonstrated *in vivo* (Pincus and Pearlman, 1943), the *in vitro* data have not been conclusive. Heller (1940) "activated" estrone by incubation with rat uterus slices and with cyanide inhibited liver slices. He suggested that the increased biological activity was due to the conversion of estrone to estradiol. Schiller (1945) using partitioning methods, demonstrated biological activity in the non-ketonic fraction of the "weak" phenols after perfusion of estrone through rat liver. He considered this material to be estradiol. The data in Table 5 demonstrate the conversion of estrone to estradiol by rat liver.

No attempt has been made in the present study to investigate the question of conjugation of estrogens by the liver but past work (Heller, 1940; DeMeio *et al.*, 1948; Schiller, 1945) has indicated that hydrolysis does not increase recovery of estrogens measured biologically. The question of *in vitro* conjugation requires further study.

The problem of the site of conversion of estradiol or estrone to estriol has not been settled. Schiller (1945) reported the recovery of estrogenic activity in the "strong" phenolic fraction after perfusion of rat liver with estrone and estradiol but this observation has not been confirmed.

Metabolism of Estrogens by Cirrhotic Liver and Hepatoma: Cirrhotic liver and hepatoma nodules retain the capacity to metabolize estradiol to estrone. Microscopic sections of these tissues demonstrated them to be typical histologically, hence contamination with normal liver cannot be invoked to explain metabolic activity. It is apparent that these diseased tissues retain enzyme systems capable of oxidizing estradiol, albeit to a lesser degree than normal tissues. When the hormone concentration was decreased sufficiently (0.006), the differences between normal and diseased tissues diminished. Again it should be noted that these conclusions are based on a small series in which there was a high degree of variability. In one experiment, hepatoma did not effect the conversion of estrone to estradiol.

Anaerobic Experiments: When estradiol or estrone was incubated with rat liver slices in evacuated Thunberg flasks or in an atmosphere of nitrogen, interconversion was observed but the recovery of added steroid was much higher than in the aerobic experiments. This observation is in disagreement with DeMeio *et al.* (1948) who reported no "inactivation" in nitrogen. Actually, his data revealed 9 to 17% inactivation but this was, as he stated, within the limits of error of the bioassay method. On the other hand, Levy (1947) reported 30–50% inactivation under anaerobic conditions. The finding of metabolic change in nitrogen does not, however, discredit the theory of a DPN linked reaction since a coupled oxidation-reduction system could provide enough hydrogen acceptors to reoxidize the reduced DPN and change limited amounts of estradiol or estrone in the absence of oxygen. By analogy with anaerobic glycolysis, a reduction would tend to be favored under anaerobic conditions.

SUMMARY

1. Counter-current distribution and photofluorometry have been applied to the separation, characterization and quantitative estimation of estrone and estradiol after incubation with rat liver slices.

2. The interconversion of estrone and estradiol occurs under both aerobic and anaerobic conditions.

3. Ethanol has a slight inhibitory effect on the oxidative breakdown of estrone and estradiol.

4. The extent of conversion of estradiol to estrone by normal and cir-

rhotic rat livers and hepatoma nodules depends on the hormone concentration.

Acknowledgements

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THE INTERCONVERSION OF ESTRONE AND ESTRADIOL BY HUMAN TISSUE SLICES[1,2,3]

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STUDIES of estrogen metabolism in animals have limited application to man because of the different metabolic pathways in different species. For example, estriol has been recovered only from human sources; epiestradiol is a product of rabbit and equine metabolism and the naphtholic estrogens are peculiar to equine species (Pincus and Pearlman, 1943). Comparative studies of the *in vitro* metabolism of estrogens by human and animal tissues have not been extensive, but species differences have been noted (Twombly and Taylor, 1942; Szego and Samuels, 1943; Samuels and McCauley, 1946). The biochemical interrelationships between steroid hormones and cellular function is at present a matter of intense interest. In order to measure directly the metabolic activity of normal and neoplastic tissues of man, an investigation of estrogen metabolism in such tissues was undertaken. The data revealed that the interconversion of estrone and estradiol as well as the further degradation of both of these compounds can be accomplished by a variety of normal and diseased human tissues.

A previous report (Ryan and Engel, 1953) described the application of counter-current distribution and photofluorometry to the separation, characterization and quantitative estimation of estrogens after incubation with rat liver slices. These same procedures have now been applied to the study of the metabolism of estrone, estradiol and estriol by human tissues.

METHODS

Tissue Preparation: All tissues were operative specimens which were obtained under aseptic conditions and kept on ice for a few minutes until ready for incubation. Slices

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[2] A preliminary report of this work was presented at the Second International Congress of Biochemistry, Paris, France, July 21–26, 1952.

[3] This is publication #778 of the Cancer Commission of Harvard University.

[4] This paper is abstracted from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine with Honors in Biological Chemistry.

were cut on a Stadie-Riggs microtome to 0.5 mm. thickness, blotted dry with filter paper and weighed. Sections were made for histologic diagnosis. In all cases, control specimens of tissue were extracted and analyzed fluorometrically. No contaminating estrogens were found.

Hormone Preparation: The specifications of the estrone and estradiol, the buffer medium and the techniques of incubation, extraction and analysis have been described (Ryan and Engel, 1952). Ethanol solutions of the hormones were used throughout and the concentrations of the steroids in the incubations were 0.01 and 0.02 mg. of hormone /mg. tissue/ml. buffer. The amounts of hormone ranged from 450–1020 μg.

Estriol[5] was freed from a small amount of contaminating estrone (1.2%) by an eight transfer counter-current distribution in 50% MeOH/CCl_4. The stock solution was prepared in absolute ethanol at a concentration of 5 mg./ml.

TABLE 1. CONVERSION OF ESTRADIOL TO ESTRONE BY HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estradiol concentration*	% Estradiol recovered	% Estrone yield
Placenta	Term	Air	.01	33	38
Placenta	4 month	Air	.01	31	11
Ileum	Normal	Air	.01	35	11
Liver	Cholangitis	Air	.02	62	5.9
Liver	Cholangitis	Air	.02	51	4.6
Liver	Cholangitis	N_2	.02	60	2.1
Adrenal	Hyperplastic	Air	.02	56	2.3
Adrenal	Hyperplastic	N_2	.02	55	0.56
Adrenal	Cushing's	Air	.02	57	1.0
Breast	Pregnancy	Air	.01	43	2.3
Breast	Pregnancy	N_2	.01	61	3.1
Breast	Fibroadenoma	Air	.01	63	1.3
Breast	Fibroadenoma	N_2	.01	82	0.56
Breast	Adenocarcinoma	Air	.02	50	0.60
Endometrium	Hyperplastic	Air	.02	75	1.7
Endometrium	Hyperplastic	N_2	.02	61	0.92
Myometrium	Fibroid	Air	.02	54	0.66
Myometrium	Fibroid	N_2	.02	61	0.45
Testes	Normal	Air	.02	71	0.50

* Hormone concentration = mg. Estradiol/mg. tissue/ml. buffer.

RESULTS

The results are presented in Tables 1 to 3. All tissues studied were found to have the ability to metabolize estrogens, but there are differences in the types and extents of the reactions. Total recoveries varied from 42 to 92% depending on the hormone used, the tissue with which it was incubated and the gas phase under which the experiment was conducted. Metabolic activity was observed under anaerobic conditions. Similar findings have been reported for rat liver (Ryan and Engel, 1953). Tissues of the digestive, reproductive and endocrine systems of man can convert estradiol to estrone. The 18–58% of the hormones unaccounted for represents conversion to as yet unknown metabolites, in addition to loss during extraction and analysis. This latter loss has been found to be approximately 10% of the starting material (Ryan and Engel, 1953).

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[5] We wish to thank the Schering Corporation and Parke, Davis and Company for the gifts of the estriol samples.

Estriol was recovered almost quantitatively after incubation with testis and term placenta. Four month placenta gave a lower recovery of estriol (Table 3). In none of these three experiments was estrone or estradiol found after incubation of estriol.

TABLE 2. CONVERSION OF ESTRONE TO ESTRADIOL BY HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estrone concentration*	% Estrone recovered	% Estradiol yield
Testes	Normal	Air	.02	47	7.0
Placenta	4 month	Air	.01	64	1.4
Placenta	Term	Air	.01	59	0
Kidney	Hydronephrosis	Air	.02	65	0.69
Endometrium	Hyperplastic	Air	.02	62	0.51
Adrenal	Hyperplastic	Air	.02	43	0.46
Adrenal	Hyperplastic	N_2	.02	62	0
Breast	Pregnancy	Air	.01	72	0.34
Myometrium	Fibroid	Air	.02	80	0

* Hormone concentration = mg. Estrone/mg. tissue/ml. buffer.

TABLE 3. INCUBATION OF ESTRIOL WITH HUMAN TISSUE SLICES

Tissue	Pathology	Gas phase	Estriol concentration*	% Estriol recovered
Testes	Normal	Air	.02	92
Placenta	Term	Air	.01	92
Placenta	4 month	Air	.01	67

* Hormone concentration = mg. Estriol/mg. tissue/ml. buffer.

DISCUSSION

Except in the cases of the testes, term placenta and ileum, the tissues used were abnormal histologically and/or functionally, therefore, the estrogen metabolism of these tissues is not necessarily comparable with their normal counterparts.

Normal Tissue: The testis, in marked contrast to the other tissues studied, converts estrone to estradiol to a greater extent than the reverse reaction. A possible explanation for this phenomenon is that the enzyme systems ordinarily involved in testosterone synthesis from Δ^4-androstene-3,17-dione are accomplishing the estrone-estradiol conversion. Dorfman (1948), Clark and Kochakian (1947), and Kochakian *et al.* (1952) have shown an intimate relation between androstenedione and testosterone and suggested that androstenedione may be a precursor of testosterone. The reaction concerned is the reduction of the 17-ketone group of androstenedione to the 17β-hydroxyl group of testosterone. It is possible that the 17-ketone group of estrone is reduced to the 17-hydroxyl group of estradiol by the same enzyme system.

One incubation of estradiol with terminal ileum resulted in an 11% yield of estrone (Table 1). This yield is higher than that found with any tissue other than placenta.

Placental tissue has by far the greatest activity of all the tissues studied, 11 and 38% yields of estrone being realized with four month and term placental tissue respectively. However, the reduction of estrone to estradiol by these tissues is minimal (Tables 1 and 2).

Abnormal Tissues: In confirmation of the work of Twombly and Taylor (1942), Samuels and McCauley (1946) and more recently Tagnon *et al.* (1952), the degradation of estradiol by human liver slices has been demonstrated. In addition, it has been shown here that this tissue can oxidize estradiol to estrone as well as degrade both to unrecognizable metabolites (Table 1).

Endocrine System: Adrenal cortex slices can effect the interconversion of estrone and estradiol (Tables 1 and 2). This is not surprising in view of the intense activity of this tissue in steroidogenesis.

Slices of human pregnancy breast, mammary fibroadenoma and adenocarcinoma (grade iii) can convert estradiol to estrone with comparable facility. Hyperplastic endometrium and uterine fibroid tissues have a similar activity. This is in contrast to the findings of Szego and Samuels (1943) who reported no conversion of estrogens by endometrial tissue taken from a woman in the third month of pregnancy. In one experiment, kidney tissue converted estrone to estradiol.

Under the experimental conditions employed, estriol does not seem to be a significant metabolic conversion product of either estrone or estradiol in any of the tissues studied. Moreover, testis and term placenta do not appear to have the capacity to cause destruction of estriol; hence, if it were formed by these tissues in any significant quantity it should have been detectable. The techniques used permit the detection and characterization of a minimum of 5 μg. of estriol.

The demonstration that a wide variety of human tissues can metabolize estrogens requires that the concept of the liver as the only and/or chief site of estrogen metabolism be questioned. It appears that such organs as breast, uterus, small bowel, testis, adrenal cortex, kidney and placenta are capable of metabolizing significant amounts of estrone and estradiol, and thus must be included in any consideration of the over-all metabolism of these compounds.

SUMMARY

1. A variety of normal and abnormal human tissues have the capacity to

interconvert estrone and estradiol and to degrade both to unrecognizable metabolites.

2. No conversion of estrone or estradiol to estriol was observed.

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INHIBITION OF DEVELOPMENT OF DCA HYPERTENSION BY A NEW SYNTHETIC STEROID

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IT HAS been well established that desoxycorticosterone acetate (DCA) administered to normal rats or to rats sensitized by high sodium intake and/or uni-nephrectomy will produce renal and vascular pathology accompanied by arterial hypertension (Selye, Hall, and Rowley, 1943; Friedman, Polley, and Friedman, 1948; Masson, Corcoran, and Page, 1949). Although the etiology of DCA hypertension has not been explained in a manner satisfactory to all workers in the field, there have been searches for agents which will either reverse an established DCA hypertension or prevent the development of DCA hypertension in experimental animals, both because of the possible clinical implications and because the use of such agents would be helpful in research on mechanisms in vascular disease.

Agents which have been reported to be effective in preventing the development of DCA hypertension in rats are: *Lipo-Adrenal Cortex*,[1] estradiol[2] (Friedman and Friedman, 1950), cortisone acetate[2] (Friedman, Friedman, and Nakashima, 1950), and Phenergan (Guillemin and Fortier, 1951).

Agents which have been without inhibitory effect on development of DCA hypertension are Compound S acetate (Masson, Corcoran, and Page, 1950), progesterone, testosterone, hyaluronidase, "acetoxy-pregnenolene," "saturated 12-keto-DCA" (Friedman and Friedman, 1950), and methylandrostenediol (Skelton, 1952).

The present paper presents data on a new steroid, "U-0160," 3,21-diacetoxy-5,7,9, pregnatrien-20-one maleic anhydride adduct (Levin,

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[1] Registered Trade-Mark, The Upjohn Company, for cottonseed oil solution of hog adrenal extract.

[2] The authors cited do not feel that the anti-hypertensive action of estradiol and of cortisone acetate represent true antagonism to the "cardiovascular-renal effects of DCA" because of the debilitation of the animals receiving them. Cortisone, in their experiments, did not prevent the cardiac and renal enlargement produced by DCA. It did, however, partially prevent the DCA-induced rise in blood pressure. The effect of estradiol was termed "non-specific," although it alleviated the hypertensive effect of DCA very markedly.

Spero, McIntosh, Wesner, Meinzer, Searcy, and Thompson, 1951) which has the ability to prevent the blood pressure changes which accompany administration of DCA to a sodium-sensitized, uni-nephrectomized rat. The structure of this compound is shown in Figure 1.

METHODS AND MATERIALS

A. *Animals.* All experments were carried out on young male rats of the Upjohn strain (a colony developed from Sprague-Dawley stock), 120 to 140 grams body weight at the beginning of the experiment. The rats were unilaterally nephrectomized (left kidney) and were given a diet of Archer dog food and 1% sodium chloride drink. Beginning on the day following nephrectomy and continuing until the end of the experiment, all animals were injected with (A) 2 mg DCA, (B) 2 mg DCA plus 2 mg U-0160, (C) 2 or 4 mg U-0160, or (D) cottonseed oil only. All preparations were made so that the daily dose was contained in 0.2 ml cottonseed oil. This concentration exceeds the solubility of U-0160, and the resulting product was a finely-divided suspension.

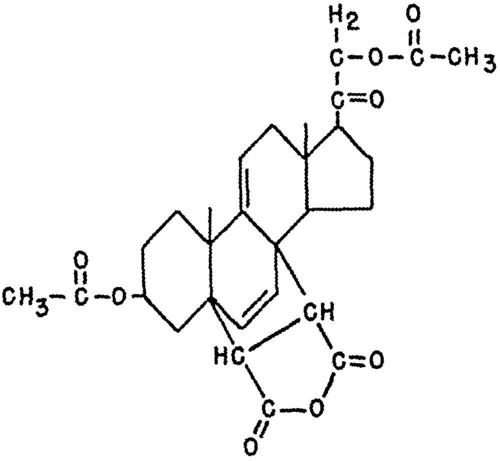

FIG. 1. Structural formula of U-0160.

All animals were injected once daily for 16 or 17 days. All rats' blood pressures were estimated three times during the experiments: The first two sets of determinations were taken to accustom the animals to the apparatus (see below for description of blood pressure estimation technique), and to trace the course of blood pressure changes. The definitive values, however, are those obtained on the seventeenth to eighteenth days, the day before sacrifice of the rats.

The rats were killed with chloroform and autopsied on the day following the last blood pressure estimation. The whole animal, the cardiac ventricles, the kidney, and, in some animals, the adrenal and thymus glands were weighed. Careful attention was given to the presence or absence of gross lung lesions, as it has been found that the occurrence of infected lungs is associated with highly aberrant blood pressures.

All experiments were designed with five or six animals in a single dosage group. Due to the occurrence of lung lesions in a number of animals and the necessity for discarding the contributions of these animals to the data, the results reported below include a number of groups of three or four rats.

Essentially the same experiment was performed at three widely separated times, as described with the results presented below.

B. *Blood Pressure Estimations.* The blood pressure estimations were carried out on conscious, unrestrained rats by a method involving measurement of optical density of the right hind foot during the deflation of a pneumatic cuff wrapped around the lower leg. The apparatus was constructed in The Upjohn Company Department of Physics by Mr. C. D. Alway and Mr. D. C. Cummings, incorporating principles described by two other laboratories: Kersten, Brosine, Ablondi, and SubbaRow (1947) found that a characteristic change in light transmittance of the rat foot occurred when the descending external pressure equalled the systolic blood pressure; Olmsted, Corcoran, Glasser, and Page (1948) described an apparatus which provided simultaneous recordings of the cuff pressure and the volume of the foot measured with a strain gauge.

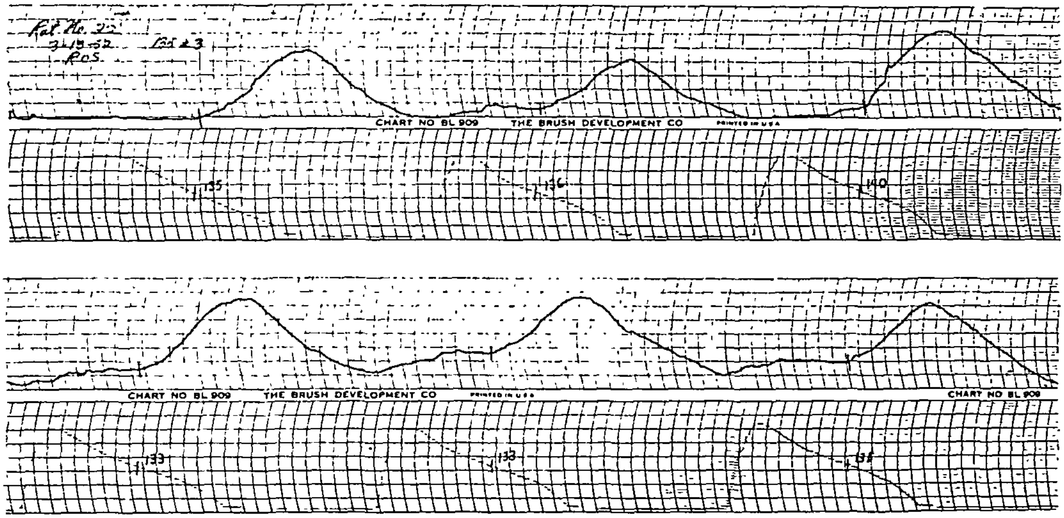

FIG. 2. Photograph of a record of blood pressure estimation with the Upjohn photoelectric tensometer. The record has been cut in half for conservation of space. The lower line indicates air pressure in the cuff, the upper line indicates photocell current. The point at which the upper line changes direction sharply is indicative of blood flow into the foot.

Our apparatus is constructed so that an intermittent light source of a specific frequency is transmitted through the foot of the lightly-held rat and impinges upon a photoelectric cell. The photo-cell current, routed through a series of amplifiers, activates the pen of a Brush Development Co. recorder. The pneumatic pressure in the bulb-cuff system is reflected by the expansion and contraction of a bellows which, through a rack-and-pinion mechanism, activates a potentiometer connected with a second pen on the Brush recorder.

The blood pressure work was all carried out in an insulated "quiet room" with extraneous noise reduced to a low level. The rats were handled as gently as possible and were placed in the rat holder in a manner designed for comfort and freedom from excessive restraint. After the first few determinations the animals were generally cooperative.

The variation in results obtained by the method is fairly large. We, therefore, rou-

TABLE 1. BLOOD PRESSURE ESTIMATIONS

Date	Treatment[2]	No. rats	Systolic blood pressures in mm Hg.[1]		
			I	II	III
4-51			*Days 5 and 6*	*Days 11 and 12*	*Day 18*
	Control[3]	6	129 (114–144)	129 (120–139)	136 (128–144)
	DCA	6	142 (118–166)	156 (138–184)	173 (153–193)
	DCA plus U-0160	6	119 (102–137)	122 (99–151)	140 (135–150)
	U-0160	6	138 (128–157)	128 (115–140)	125 (102–153)
6–51			*Days 10 and 11*	*Day 14*	*Day 17*
	Control[3]	4	127 (118–138)	133 (119–150)	130 (126–135)
	DCA	5	160 (135–202)	167 (140–197)	171 (146–197)
	DCA plus 2 mg. U-0160	5	130 (105–154)	122(110–135)	129 (115–135)
	DCA plus 4 mg. U-0160	4	126 (109–160)	130 (122–143)	133 (121–146)
	U-0160[4]	5	147 (126–190)	128 (119–140)	140 (128–169)
3-52			*Days 2 and 3*	*Day 8*	*Days 17 and 18*
	Control[3]	3	118 (101–141)	137 (123–164)	133 (127–138)
	DCA	5	112 (100–125)	154 (133–169)	167 (152–186)
	DCA plus U-0160	4	114 (98–136)	113 (105–123)	129 (120–142)
	U-0160	5	129 (120–135)	118 (94–141)	120 (101–151)

[1] Figures in parentheses indicate extremes within groups. Days indicated are days after nephrectomy.

[2] All doses 2 mg. per rat per day unless otherwise indicated.

[3] Uninephrectomy, 1% NaCl drink, cottonseed oil injections or no injections.

[4] Suspended in saline. All other preparations suspended in cottonseed oil.

TABLE 2. BODY AND ORGAN WEIGHTS[1]

Date	Treatment[2]	No. rats	Body weights	Cardiac weights	Kidney weights
4-51	Control[3]	6	179 (155–214)	324 (308–367)	705 (594– 968)
	DCA	6	160 (125–182)	442 (336–457)	823 (700– 920)
	DCA plus U-0160	6	174 (156–201)	380 (358–408)	779 (708– 860)
	U-0160	6	175 (155–218)	327 (319–324)	634 (542– 784)
6-51	Control[3]	4	171 (157–182)	308 (290–340)	640 (590– 690)
	DCA	5	187 (172–203)	379 (350–440)	882 (740–1100)
	DCA plus 2 mg. U-0160	5	131 (110–156)	336 (310–400)	974 (910–1060)
	DCA plus 4 mg. U-0160	4	151 (139–163)	355 (330–380)	1237 (1070–1380)
	U-0160[4]	5	175 (166–188)	340 (310–370)	738 (660– 910)
3-52	Control[3]	3	199 (174–220)	304 (293–320)	723 (690– 825)
	DCA	5	169 (150–190)	366 (350–405)	902 (800–1020)
	DCA plus U-0160	4	152 (128–188)	292 (202–352)	1044 (755–1185)
	U-0160	5	176 (166–188)	287 (271–298)	885 (668–1397)

[1] Organ weights expressed as mg. per 100 g. body weight. Figures in parentheses indicate extremes within groups.

[2] All doses 2 mg. per rat per day unless otherwise indicated.

[3] Uninephrectomy, 1% NaCl drink, cottonseed oil injections or no injections.

[4] Suspended in saline. All other preparations suspended or dissolved in cottonseed oil.

Note that administration of U-0176 did not antagonize the effects of DCA on blood pressure to any significant extent, nor did the administration of 2 mg per day of maleic anhydride.

The increase in renal mass caused by U-0160 is interesting, and may play a role in the blood pressure effects produced by this compound. We have insufficient data to attempt to explain the renal effects of U-0160 because the kidneys of these animals are undergoing changes which are the resultant of several factors: compensatory hypertrophy as a result of removal of the contralateral kidney, response to the sodium-DCA-induced diuresis, and response to the renomegalic effect of DCA itself. The role of U-0160 in the structure and physiology of this type of kindey is, therefore, a research problem in itself.

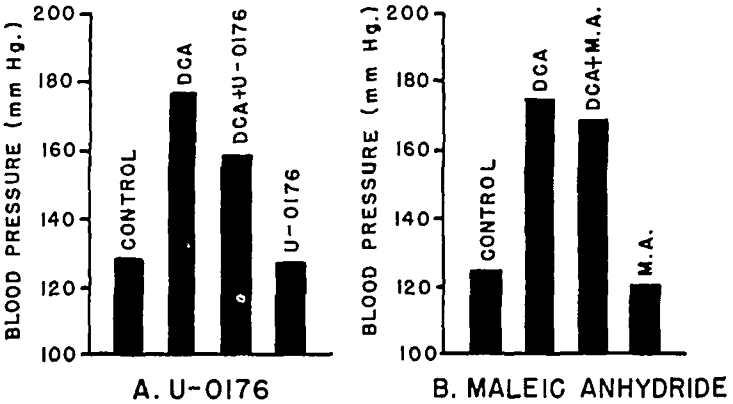

FIG. 4. A. Terminal blood pressures (Day 17–19) of rats receiving 2 mg DCA per day and/or 2 mg U-0176 per day; pooled data of two experiments similar in design to those shown in Figure 2. B. Terminal blood pressures (Day 19) of rats receiving 2 mg DCA per day and/or 2 mg maleic anhydride in aqueous solution. Single experiment, four or five rats per dose.

SUMMARY

U-0160 (3,21 diacetoxy-5,7,9-pregnatrien-20-one maleic anhydride adduct) was effective in preventing the hypertension produced by DCA in the unilaterally nephrectomized, sodium-sensitized rat. This property is not shared by U-0176 (U-0160 less maleic anhydride) or by maleic anhydride itself.

U-0160 also largely prevents the cardiac hypertrophy accompanying DCA hypertension, but augments the increase in renal mass.

It is considered improbable that the action of the compound is through release of 11-oxygenated hormones from the adrenal cortex, as there is no evidence of adrenal hypertrophy or thymic involution produced by the compound.

Acknowledgments

The authors are grateful to Dr. R. H. Levin, The Upjohn Company, Kalamazoo, Michigan, for generous supplies of U-0160 and U-0176 and for his criticism of this manuscript.

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ASSAY OF ACTH ACTIVITY BASED ON THE EOSINOPENIC RESPONSE IN NORMAL AND HYPOPHYSECTOMIZED MICE[1]

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SINCE 1937, a number of assay procedures for ACTH have been proposed. These have generally been based upon a change in size of the adrenal glands of normal rats and baby chicks, or changes in the adrenal glands of hypophysectomized animals. The hypophysectomized rat has been the animal of choice, and the effect of ACTH on adrenal repair, maintenance, ascorbic acid or cholesterol levels have been used as end points in the assay. The ascorbic acid depletion test proposed by Sayers, *et al.* (1948) has proved to be the most satisfactory one. It is an extremely sensitive test, reasonably accurate and very useful in detecting small amounts of hormone in body fluids. (Gemzell, 1952.)

At the present time, the role of ascorbic acid in the synthesis or release of adrenal cortical hormones is unknown (Sayers, 1950). The factor in the ACTH that brings about the change in ascorbic acid level, may not be the same factor that produces an increase in size of the adrenal or release of the adrenal hormones. Reinhardt, *et al.* (1951a, b and c) compared the results of the ascorbic acid depletion test with those of the eosinopenia and adrenal maintenance tests. He found that the results do not compare favorably. A high ascorbic acid depleting power does not necessarily correspond to a high rate of secretion of the cortical hormones as measured by a reduction in eosinophils or in thymus and lymph node weights. These data have led a number of workers to suggest that in order to obtain accurate results in the assay of ACTH preparations several bioassay methods must be used (Greenspan, Li, Simpson and Evans, 1950; Selye, 1951).

In recent years a bioassay method for adrenal cortical hormones has been developed which is extremely sensitive, accurate and relatively easy to perform in mice (Speirs and Meyer, 1951; Speirs, Wragg, Bonner and Homburger, 1951). This assay is based upon the ability of the adrenal

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cortical hormones to reduce the number of circulating eosinophils in adrenalectomized mice during a three hour period. It should be possible to modify this eosinophil method to assay ACTH. The assay would then measure the ability of ACTH to release the 11-oxycorticosteroid hormone from the adrenal cortex and would thus differ from the ascorbic acid depletion, repair or maintenance tests, and could be used to supplement them. Furthermore, it would be possible to measure the duration of the ACTH action upon the adrenal releasing the hormone. Such an assay might be very useful in developing and standardizing long-acting ACTH preparations.

The following paper is a report of a series of investigations to determine if the eosinopenic response of normal mice can be used to assay long acting ACTH preparations.

MATERIAL AND METHODS

The mice used in these experiments were the BBF_1 hybrid animals developed for the assay of adrenal cortical hormones; mice of strain 129 were used for comparison. The BBF_1 mice are the offspring of a cross between C57BR/cd and C57BL/6. Only male mice approximately 6 weeks of age and weighing between 20 and 25 grams were used.

The ACTH used in these experiments was supplied by Armour and Company, and assayed by them according to the ascorbic acid procedure. These hormones were used in order to determine possible assay procedures and no effort has been made to compare the eosinopenic values of various types of ACTH.

The techniques of performing eosinophil counts described earlier (Speirs and Meyer, 1949; Speirs, 1952a) have been modified slightly. Standard WBC diluting pipettes were used and the cells were counted in an Eosinophil Counting Slide (Speirs, 1952b) using a bifocal microscope containing 15× wide field oculars and 16 mm. objective. The eosinophil diluting fluid consisted of the following:

Phloxine B	0.02	gm.
Diethylene Glycol	2	ml.
Alconox (or similar detergent)	0.002	gm.
Distilled Water	85	ml.
Acetone, added just before using	15	ml.

Precautions were taken in order to avoid an eosinopenia due to stress. To avoid disturbances due to fighting, etc., the mice were kept in individual cages which were placed in a dark cabinet maintained at 27° C. for at least 24 hours prior to the experiment. Food, consisting of Purina Fox Chow with a weekly supplement of rolled oats, and water, were given ad libitum. Eosinophil counts were performed within 10 minutes after the animals were first disturbed. They were removed from their cages and placed in a battery jar containing wood chips and warmed by a 100 watt lamp. As soon as the animals became active, their tails were washed in warm soapy water, rinsed, and a small nick made through the skin and through one of the prominent blood vessels. Profuse bleeding occurred in all cases. The first two drops of blood were discarded, and fresh blood was drawn into the WBC pipette and diluted 1:20 with the above-mentioned diluent. One pipette of blood was taken from each animal, and from that two chambers on the Eosinophil Counting Slide were loaded. After allowing the cells to settle and stain for approximately 15 minutes, the eosinophils in both chambers were counted. The total count of both chambers (twenty $1 \times 1 \times 0.2$ mm. volumes) was multiplied by 5 to obtain the number of cells per cu. mm. of blood.

The changes in circulating eosinophils were given in this paper as percent decreases. They were computed as follows:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1\right) \times 100.$$

All positive values (an increase in eosinophils) were recorded as zero percent decreases.

PROCEDURE AND RESULTS

In designing any assay procedure involving the fluctuation of eosinophils in normal animals, one must first consider the normal variation of these cells, particularly the diurnal variation (Halberg, 1951). In order to determine this in our animals, normal male mice were maintained in individual cages and kept in a dark cabinet with ample food and water. These mice were considered to be relatively undisturbed or free of stress prior to the experiment. At three hour intervals throughout the day and night, groups of animals were removed from their cages without disturbing the remaining animals, and eosinophil counts were performed. Each animal was used for only one eosinophil determination. This was repeated on two separate occasions and a total of 12 eosinophil counts were obtained for each period. The mean eosinophil counts and the standard error of the mean are plotted in Figure 1.

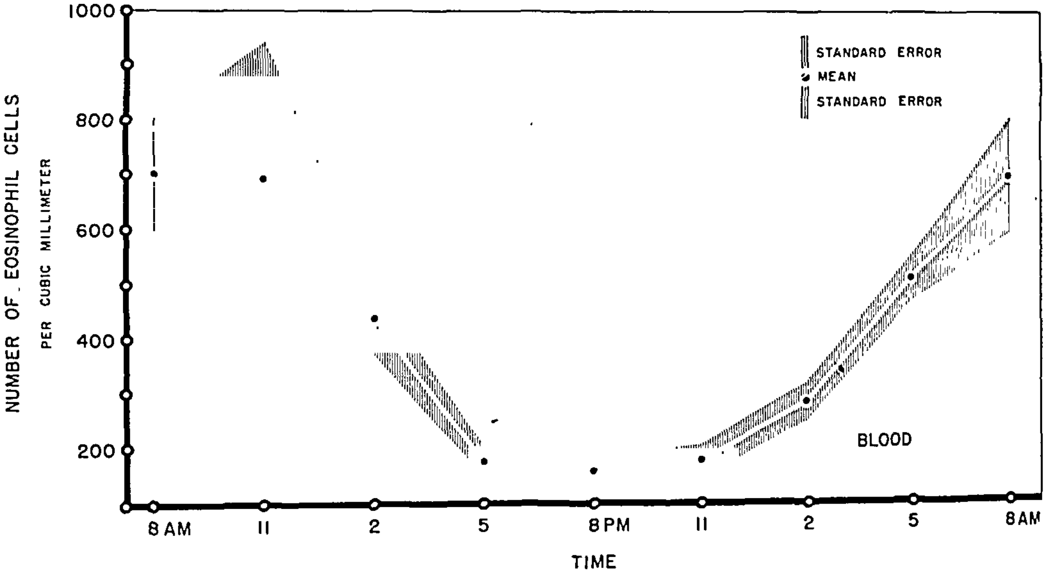

FIG. 1. Diurnal Variation of Eosinophil Cells in Blood. Each point represents the mean and standard error of 12 normal male BBF_1 mice.

It may be seen that the number of eosinophils fluctuates greatly during the day. A peak of 700 cells per cu. mm. was obtained at 8 and 11 A.M. and a low of 155 cells occurred between 5 and 11 P.M.

The next factor to consider is the effect of mild stress upon the number of circulating eosinophils. Figure 2 illustrates the mean and standard error of the number of eosinophils in normal C57 Brown mice following mild stress such as handling (from Speirs and Meyer, 1949). A similar eosinopenia was also obtained in the BBF_1 mice (unpublished data). Thus an eosinopenia occurs in these animals following mild stress. There is complete recovery within 24 hours after the animals are submitted to a single mild stress.

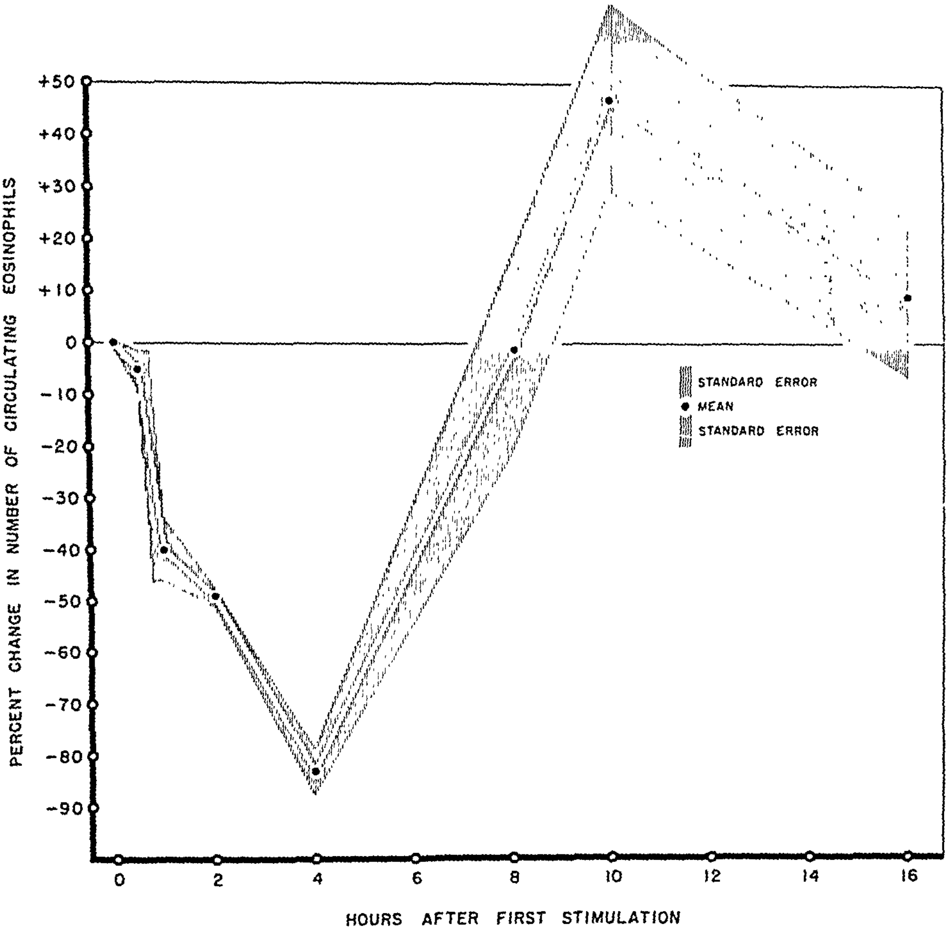

Fig. 2. Variation in the Number of Circulating Eosinophils in Normal Male C57 Brown Mice Following Mild Stress. Each point represents the mean and standard error of 9 mice.

Figure 3A shows the response curve of mice which had received subcutaneously 2 I.U. of ACTH in $\frac{1}{4}$ cc. of saline. This curve is essentially the same as the one obtained following a mild stress. By 10 hours recovery had begun and was complete by 18 to 24 hours.

Figure 3B presents results obtained following the subcutaneous injection of 2 I.U. of ACTH suspended in 0.1 cc. of gelatine. It may be seen that in the 6 and 10 hour groups, there was a decrease in eosinophils of 98% and 99%. Recovery began by 18 hours and was complete by 24 hours.

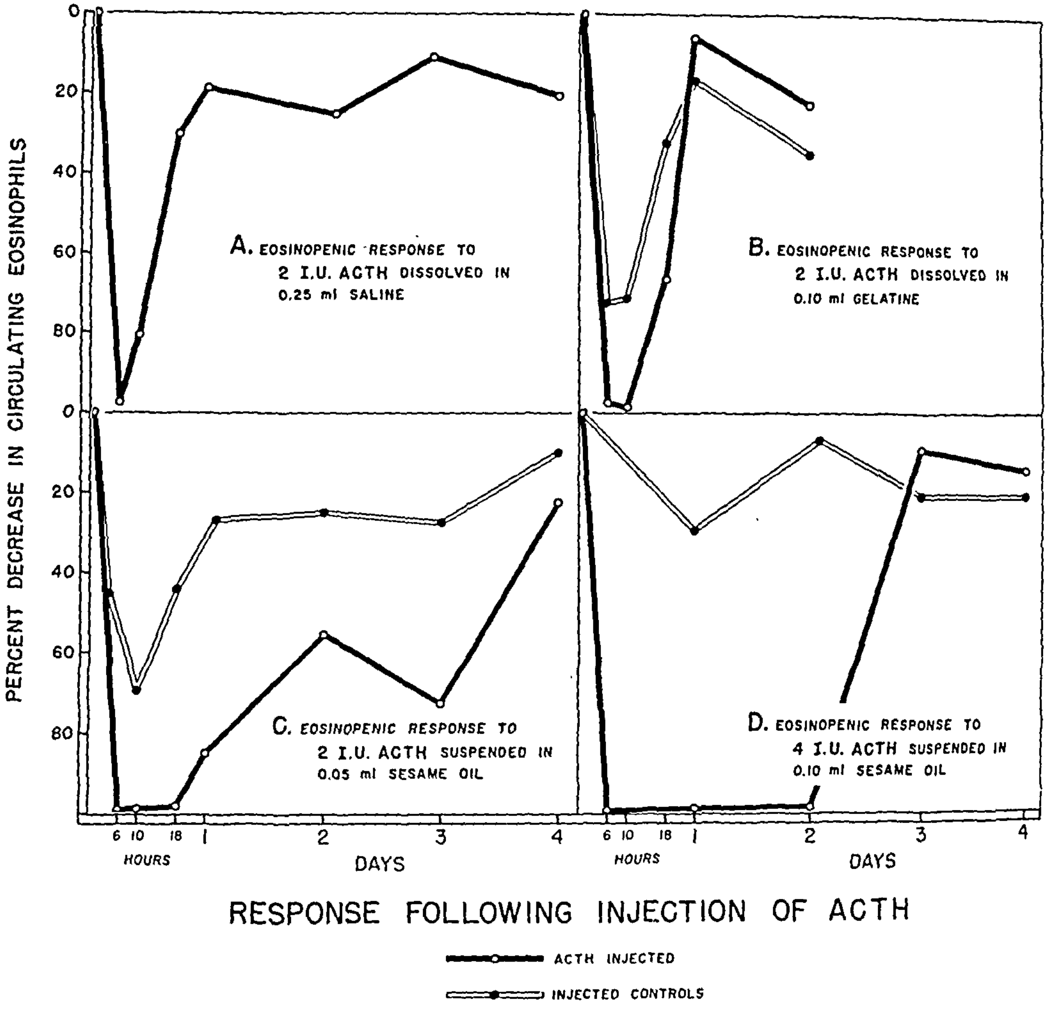

FIG. 3. Eosinopenic Response of Normal Male BBF₁ Mice to ACTH. Each point represents a mean obtained from at least 6 mice.

Figures 3C and D show animals which were injected with the ACTH suspended in sesame oil. In the group receiving 2 I.U., there was a complete absence of eosinophils 6 hours after the injection, and a 99% and 98% decrease in the 10 and 18 hour groups. Twenty-four hours following the injection an eosinopenia of 85% was found. Recovery began by 48 hours. In the group receiving 4 I.U. of ACTH suspended in sesame oil recovery did not occur until 72 hours following the injection.

It should be noted that in all the animals which received control injections, complete recovery in the number of circulating eosinophils occurred within 24 hours. This agrees with earlier published data in which the effects of epinephrine and mild stress were studied (Speirs and Meyer, 1949). However, the ACTH suspensions in gelatine and oil produced an eosinopenia which persisted for 24 hours or more. These data suggested that possibly an assay for ACTH could be developed in which only the 24 hour response was considered.

TABLE 1. 24 HOUR EOSINOPENIC RESPONSE OF NORMAL MALE BBF_1 MICE TO ACTH DISSOLVED IN GELATINE

VOLUME INJECTED	I.U. ACTH	MICE	PERCENT DECREASE IN EOSINOPHILS (0–100)
0.25 ml.	0.5	6	50 ± 11.1
	1.0	6	59 ± 6.5
	2.0	5	95 ± 2.1
	3.0	6	97 ± 1.3
	5.0	6	99 ± 0.4
0.5 ml	0.5	6	58 ± 11.8
	1.0	6	55 ± 9.5
	2.0	6	96 ± 1.0
	4.0	5	100 ± 0.4
	10.0	6	100 ± 0
0.1 ml	2.0	12	6 ± 3.1
0.25 ml	2.0	5	95 ± 2.1
0.5 ml	2.0	6	96 ± 1.0
0.25 ml	0	6	25 ± 9.3 CONTROLS
0.5 ml	0	6	51 ± 13.6 CONTROLS

Table 1 summarizes the data in a group of 76 mice which had received various gelatine suspensions of ACTH. In the first group the ACTH was suspended in 0.25 ml. of gelatine. It may be seen that 0.5 I.U. of ACTH produced an average eosinopenia of 50%, 24 hours after the injection. One I.U. produced a slightly greater response, and 2, 3 and 5 I.U. produced 95%, 97%, 99% decreases respectively. The same responses were obtained when the ACTH was suspended in 0.5 ml. of gelatine. However, if the volume of gelatine was reduced to 0.1 ml., no significant eosinopenia occurred. The animals which had received 0.25 and 0.5 ml. showed an average fall in eosinophils of 25% and 51% respectively. It appears that the larger quantities of gelatine (0.5 ml.) produce a stress reaction which lasts throughout the 24 hour period.

Table 2 shows results obtained following the injection into 120 mice of ACTH suspended in sesame oil. It may be seen that a graded dose response is obtained when the ACTH is suspended in 0.05 ml. of oil. Similar results were obtained when a volume of 0.1 ml. was injected, but as the volume was increased to 0.2 and 0.4 ml. responses became less pronounced. The control mice which had received 0.4 ml. of oil had an eosinopenia equal to the group that had received the same volume containing 2 I.U. ACTH. It would appear from the data presented here that the smaller volume of the vehicle produced the most consistently high response.

TABLE 2. 24 HOUR EOSINOPENIC RESPONSE OF NORMAL MALE BBF_1 MICE TO ACTH SUSPENDED IN SESAME OIL

VOLUME INJECTED	I.U. ACTH	MICE	PERCENT DECREASE IN EOSINOPHILS 0 20 40 60 80 100
0.05 ml.	0.25	4	6 ± 6.5
	0.5	4	13 ± 13.5
	1.0	10	44 ± 9.2
	2.0	12	92 ± 3.4
0.1 ml.	0.25	9	6 ± 5.6
	0.5	9	28 ± 9.2
	1.0	12	30 ± 11.5
	2.0	8	86 ± 12.5
	4.0	9	89 ± 11.1
0.05 ml.	2.0	12	92 ± 3.4
0.10 ml.	2.0	8	86 ± 12.5
0.20 ml.	2.0	6	68 ± 12.9
0.40 ml.	2.0	6	48 ± 18.1
0.05 ml.	0	6	▨ 31 ± 8.2
0.1 ml.	0	6	▨ 29 ± 10.5 CONTROLS
0.2 ml.	0	6	▨ 11 ± 6.6
0.4 ml.	0	3	▨ 49 ± 24.9

Table 3 shows the statistical treatment of the data obtained when two strains of mice were injected with 1 and 2 I.U. of ACTH suspended in $\frac{1}{4}$ ml. of gelatine. The first line shows results obtained when two groups of BBF_1 mice were injected with ACTH. The theoretical potency was 100%, the potency ratio obtained was 98.9%, with a standard error of 30%. The index of precision was 0.33 for the 6 sets, indicating a reasonably high degree of accuracy. In line 2 are the results obtained when 11 pairs of mice from each of two strains are compared. In this case the sensitivity ratio was 96% with a standard error of 11%. The index of precision was 0.16, indicating a very high degree of accuracy.

Injections of ACTH and gelatine were made into mice at periods varying from 4 to 30 days after hypophysectomy. A regression formula of $Y = 68 + 67.9 \text{ Log } X$ was obtained for 21 mice injected with $\frac{1}{2}$, 1 and 2 I.U. of ACTH in gelatine. Approximately the same sensitivity was obtained as in normal mice.

TABLE 3. STATISTICAL RESULTS OBTAINED WHEN STRAIN BBF_1 AND STRAIN 129 MICE ARE INJECTED WITH 1 AND 2 I.U. ACTH IN 0.25 ML. GELATINE

COMPARISON OF BBF_1 AND 129 MICE FOR ASSAY OF ACTH

MICE COMPARISON	N	RATIO OF DOSES OF ACTH I.U.	b SLOPE	S D	λ	POTENCY RATIO ± S E %	t SLOPE
BBF_1 vs BBF_1	6	$\frac{2}{1}$	− 160	53.3	0.33	98 ± 30	.13
BBF_1 vs 129	11	$\frac{2}{1}$	− 121	20.9	0.16	96 ± 11	.56

Injections of 2 mg. growth hormone in gelatine or sesame oil did not produce a significant eosinopenia over a 24 hour period. On the other hand injections of very toxic oils such as turpentine rsulted in an eosinopenia which persisted for 24 hours or longer. This eosinopenia occurred even in the hypophysectomized animals. For example, injections of 0.05 ml. of turpentine into 7 hypophysectomized animals resulted in the death of 4 animals and an average eosinophil decrease of 76% in the remaining 3.

DISCUSSION

In this paper an attempt has been made to determine the various factors which would be involved in the assay of ACTH in intact mice. Earlier experiments had indicated that a decrease in the number of circulating eosinophils in a relatively short period of 3 or 4 hours was a specific response to the 11-oxycorticosteroids. An assay which is based upon the decrease in eosinophils following an ACTH injection would therefore measure the amount of 11-oxycorticosteroid hormone released from the adrenal cortex. However, there are a number of factors which make the 4 hour assay impractical in the mouse. Intact animals which have been subjected to handling or mild stress will show an eosinopenia even without the ACTH injections. Furthermore there is a great diurnal variation which must be taken into account. Animals which have been hypophysectomized will also respond to handling and epinephrine injections with a slight eosinopenia (Speirs and Meyer, 1949). In addition it is very difficult to obtain two blood samples from these animals within a period of 4 hours.

However, it was noted in the experiments reported in this paper that following mild stress or injections of non-toxic solvents the eosinophils would completely recover by 16 to 20 hours. The diurnal variation was also found to be consistent. On the other hand, injections of ACTH suspended in gelatine and oil produced an eosinopenia which persisted for 24 hours or longer. These factors suggested that an assay could be devised which would measure the ability of ACTH to depress the number of circulating eosinophils during a 24 hour period.

Injections of 0.5, 1 and 2 I.U. of ACTH were made into intact mice and the following regressions were obtained:

$$Y = 56.02 + 138 \text{ Log } X \text{ (in 0.25 ml. gelatine)}$$
$$Y = 51.1 + 133 \text{ Log } X \text{ (in 0.05 ml. sesame oil).}$$

Determinations of the accuracy of the method were also made (Table 3). These analyses indicated that the assay was reasonably accurate and repeatable with as few as 6 sets of animals. Further comparisons were made between the BBF_1 and the 129 strains. The first strain is known to be highly sensitive to small amounts of 11-oxycorticosteroids and, when adrenalectomized, has been found to be the most satisfactory strain to use for the assay of corticoids. BBF_1 animals are approximately 12 times more sensitive to cortisone acetate than the 129 mice. On the other hand, the 129 strain has been shown to be less responsive to mild stress and handling as measured by a decrease in the circulating eosinophils. It was an entirely unexpected observation that these two strains responded in a similar manner to the long acting ACTH. The 129 mouse is much more docile and easier to handle than the BBF_1 and would therefore make a better assay animal. Other strains are being investigated to determine their sensitivity to ACTH and 11-oxycorticosteroids.

The assay of ACTH by the eosinophil procedure as described above is far less sensitive than the ascorbic acid depletion procedure of Sayers (1948). However, it has the distinct advantage of determining qauntitatively the amount of release of the 11-oxycorticosteroids from the adrenal and the approximate period of time over which the release occurs. For example, very high doses of ACTH in water solutions have little or no eosinopenic action 24 hours after injection. However, if the ACTH is suspended in gelatine or sesame oil a marked eosinopenia occurs. An assay based upon the duration of eosinopenia would thus be very useful in standardizing and comparing long acting preparations.

Preliminary experiments have indicated that hypophysectomized mice may also be used to assay ACTH solutions. These animals appear to be as sensitive to the ACTH as intact animals. The primary difficulty with this assay in intact animals is that very toxic materials may produce an eosinopenia which persists for 24 hours or longer. Injections of large volumes of slightly toxic materials which are slowly absorbed might also produce a persistent eosinopenia even in hypophysectomized mice. Therefore, care should be taken to run simultaneous controls using identical type and quantity of vehicle.

SUMMARY

The eosinophils in the circulating blood of intact, undisturbed mice were found to have a marked diurnal variation. When counts were performed on groups of 12 mice, an average of 700 eosinophils per cu. mm. was obtained between 8 and 11 A.M. with a rapid decrease to a low value of 155 cells per cu. mm. by 8 P.M. Complete recovery occurred by 8 A.M. the following morning.

Mild stress produced a marked eosinopenia in 3 to 6 hours followed by an eosinophilia. There was a recovery in the level of circulating eosinophils within 24 hours.

Injections of ACTH in saline solution produced an eosinopenia which was almost identical to that produced by mild stress. However, when the ACTH was suspended in gelatine or sesame oil, the period of eosinopenia was prolonged up to 24 hours or more.

These observations suggested that an assay for ACTH could be performed in either intact or hypophysectomized mice. The per cent decrease in eosinophils over a 24 hour period was found to be proportional to the quantity of ACTH injected. Statistical analysis of the data indicated that the regression was $Y=56.02+138\,X$ when 0.5, 1 and 2 I.U. of ACTH was suspended in 0.25 ml. gelatine and injected into intact mice. The index of precision of 6 sets of animals injected with 1 and 2 I.U. ACTH in gelatine was found to be 0.33.

Two strains of mice tested for this assay, the BBF_1 hybrids and the 129 mice, were found to give almost identical responses. The index of precision of 11 sets of animals was found to be 0.16.

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THE ACTION OF DESOXYCORTICOSTERONE ACETATE ON THE MAMMARY GLAND OF THE IMMATURE OVARIECTOMIZED RAT

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DESOXYCORTICOSTERONE acetate (DCA) exerts a progesterone-like action on the uterus (Van Heuverswyn, *et al.*, 1939a; Engle, 1941; Masson, 1943) and mammary gland (Mixner and Turner, 1942; Trentin and Turner, 1948; Speert, 1940) of several laboratory animals. It also shows estrogen-like activity on the mammary gland (Van Heuverswyn, *et al.*, 1939b; Leonard and Reece, 1942.) However, no comprehensive study of its action on the mammary gland of the rat has been undertaken. The experiments reported here were designed to elucidate the action of DCA on the mammary gland of the immature ovariectomized rat, alone, and in combination with estradiol or progesterone.

MATERIALS AND METHODS

Twenty-four day-old female albino rats were ovariectomized. Treatment was begun on the 29th day with daily subcutaneous injections of the hormones in sesame oil with the exception of the highest dose of DCA which was administered in propylene glycol.[3] A standard daily dose of 0.1 γg. estradiol and of 4.0 mg. progesterone was injected. The doses of DCA were varied from 1.0 to 10.0 mg. daily. The rats were treated for 12 days. Autopsy was performed the day after the last injection. The right abdominal-inguinal mammary glands of each animal were removed, fixed in Bouin's solution, stained with Mayer's haematoxylin, and stored in methyl salicylate for later study as whole mounts.

The areas of the glands were traced onto weighing paper and later measured with a Keuffel and Esser planimeter. The glands were trimmed of peripheral fat and connective tissue and weighed on a triple-beam balance. Lobulo-alveolar growth was rated on a similar scale. The uteri were also trimmed, split, blotted on bibulous paper, and weighed for comparative study.

RESULTS

Daily doses of 2 and 5 mg. DCA resulted in glands with ducts which were dilated and extended, and buds which were expanded (Table 1). There was no alveolar formation. Mammary gland areas were somewhat smaller

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[3] The steroids used in these studies were generously supplied by the Schering Corporation, Bloomfield, New Jersey.

than in the controls. The glands resembled histologically those of animals treated with estradiol alone. At a daily dose of 10 mg., DCA had no effect on mammary duct growth. The glands resembled those of untreated castrated controls, in spite of the fact that uterine growth was almost as great as that of rats treated with estradiol.

TABLE 1. INFLUENCE OF DCA OR ESTROGEN ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.^{2})	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
None	5	64	105	6.0 ± 0.37	0.29 ± 0.070	0/5	32.2
0.1 γg. Estrogen	4	63	102	6.1 ± 0.35	0.36 ± 0.050	0/4	128.5
2 mg. DCA	3	65	110	5.0 ± 0.47	0.24 ± 0.015	0/3	Atrophic
5 mg. DCA	3	69	113	3.9 ± 0.32	0.26 ± 0.040	0/3	Atrophic
10 mg. DCA	2	50	93	5.9	0.36	0/2	93.5

Calculation of standard error of the mean:

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

It is well known that estradiol and progesterone synergize to induce lobulo-alveolar growth. Since DCA apparently exhibited estrogenic properties in the preceding experiments, further experiments were designed to determine whether DCA would substitute for estradiol in this synergistic combination (Table 2). Animals injected with 4.0 mg. of progesterone daily showed glands with some duct and end bud expansion, and dubious lobulation. In all rats receiving 0.1 γg. estradiol and 4.0 mg. progesterone daily, the ducts and end buds were expanded, and excellent lobulation occurred.

TABLE 2. INFLUENCE OF PROGESTERONE, DCA AND PROGESTERONE, AND ESTROGEN AND PROGESTERONE ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.^{2})	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
4 mg. Progesterone	3	71	114	7.8 ± 0.39	0.36 ± 0.048	1/3	53.3
4 mg. Progesterone + 2 mg. DCA	3	54	92	6.8 ± 0.58	0.28 ± 0.011	0/3	45.0
4 mg. Progesterone + 5 mg. DCA	3	62	106	6.3 ± 0.43	0.31 ± 0.020	0/3	84.2
4 mg. Progesterone + 0.1 γg. Estrogen	3	64	101	7.2 ± 0.67	0.35 ± 0.057	3/3	114.4

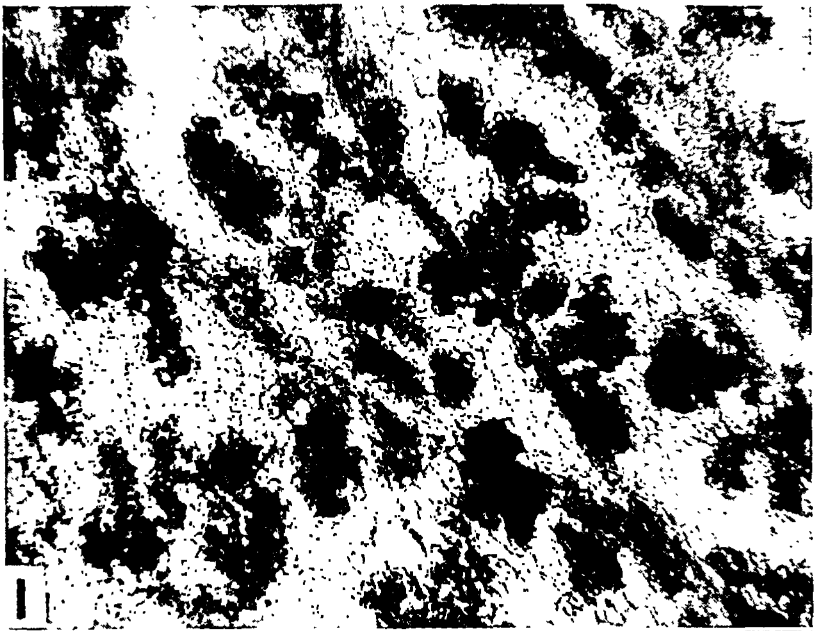
(Fig. 1). A combination of 4.0 mg. progesterone and 2 mg. DCA daily led to expanded ducts and buds, and, in two out of three rats, doubtful alveolar development. With a combination of 4.0 mg. progesterone and 5 mg. DCA daily, there was spotty duct extension and dilation, slight bud expansion, and no lobulation.

Rats given 5 mg. of DCA daily, in addition to progesterone, had uterine weights which were around 50% above rats receiving progesterone alone. Two mg. DCA failed to effect this increase. In rats treated with estradiol and progesterone, average uterine weight was more than 100% increased over the progesterone controls.

The next step was to determine whether or not DCA would exhibit any progesterone-like activity in combination with estradiol. The possibility of any additive effects with the estrogen was also looked for. Reference to Table 3 shows that 0.1 γg. estradiol and 2.0 mg. DCA daily did not elicit any lobulo-alveolar growth, while 0.1γg. estradiol and 5.0 mg. DCA led to some lobulation in four of five rats: three gave a "plus one" reaction, and one gave a "plus two" reaction. Mammary areas showed little change from those of rats receiving estrogen alone (Table 1). Estradiol, 0.1 γg., and DCA, 10 mg., daily led to a "plus three" in one and a spotty "plus two" reaction in two of five rats. One of the better responses is seen in Figure 2, comparable to that obtained with 0.1 γg. estradiol and 4.0 mg. progesterone (Fig. 1). Mammary areas were increased in this group, comparable to those of rats treated with estradiol and 4.0 mg. progesterone (compare with Table 2). Propylene glycol alone had no effect on the mammary glands, which were identical in appearance with those of the ovariectomized, uninjected rats. It is evident from these results that DCA at a dose of 5 mg. daily exhibits only a slight progesterone-like activity in the syner-

TABLE 3. INFLUENCE OF ESTROGEN-DCA COMBINATIONS ON MAMMARY GROWTH

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.²)	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
0.1 γg. Estrogen + 2 mg. DCA	4	55	94	5.1 ±0.44	0.34 ±0.041	0/4	120.3
0.1 γg. Estrogen + 5 mg. DCA	5	62	114	6.2 ±0.18	0.33 ±0.059	4/5	111.1
0.1 γg. Estrogen + 10 mg. DCA	5	42	92	7.5 ±0.63	0.31 ±0.029	2–3/5	111.3
Propylene glycol	2	—	—	Castrate Atrophy			—



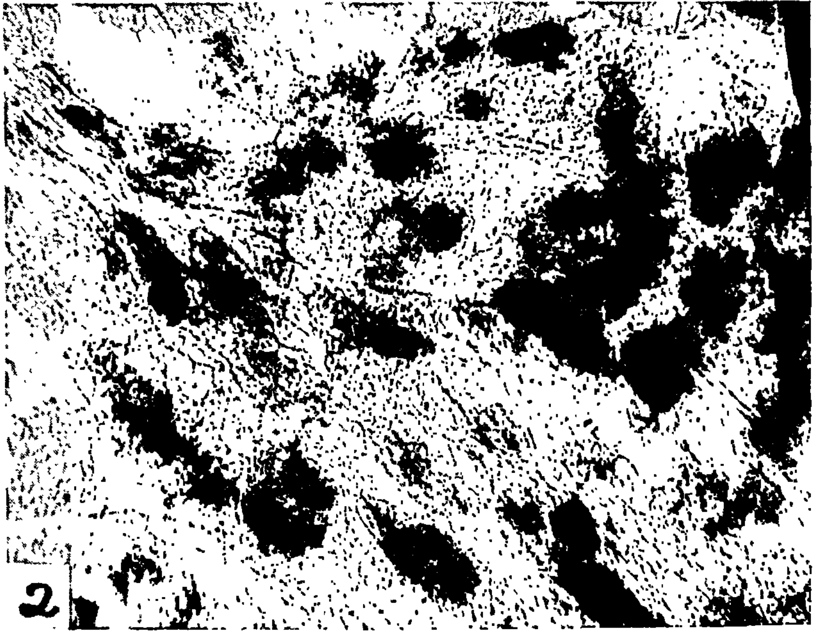


FIG. 1. Mammary gland of rat injected with 0.1 γg. estradiol and 4.0 mg. progesterone for 12 days.

FIG. 2. Mammary gland of rat injected with 0.1 γg. estradiol and 10.0 mg. DCA for 12 days.

gistic reaction with estradiol causing lobulation. Doubling the daily dose of DCA produced little additional effect on mammary lobulation. Nor could any additive effects with the estrogen be detected, either on the mammary duct system or on uterine weight.

The possibility remained that DCA, because of structural similarity to progesterone, might exhibit an additive or antagonistic action to the lobulo-alveolar induction by 4.0 mg. progesterone and 0.1 γg. estradiol daily. DCA, in doses of 1.0, 2.0, and 4.0 mg. daily failed to exert any additive or antagonistic effects, either on lobulo-alveolar growth, duct structure, or total area (compare results in Table 4 with those in Table 2). Uterine weights were unaltered by the superimposed DCA treatment.

TABLE 4. INFLUENCE OF DCA ON MAMMARY GROWTH ELICITED BY ESTROGEN AND PROGESTERONE

Daily treatment	Number of rats	Body weight (gm.)		Area (cm.^{2})	Mammary gland		
		Change	Final		Weight (gm.)	No. of alveolar responses	Uterine weight (mg.)
0.1 γg. Estrogen+ 4 mg. Progesterone +2 mg. DCA	2	77	116	6.9	0.33	2/2	111.7
0.1 γg. Estrogen+ 4 mg. Progesterone +2 mg. DCA	4	64	106	7.2±0.81	0.37±0.054	4/4	102.2
0.1 γg. Estrogen+ 4 mg. Progesterone +4 mg. DCA	4	58	101	6.8±0.60	0.33±0.038	4/4	114

DISCUSSION

DCA has mammary duct-stimulating activity in the mouse in doses of 0.5 mg. every other day (Van Heuverswyn, *et al.*, 1939b) and in the partially-hypophysectomized rat (Leonard and Reece, 1942). The present experiments furnish evidence that it exerts both an estrogen-like and a progesterone-like action on the mammary gland of the immature ovariectomized rat. Even though DCA is estrogenic when administered alone, it fails to substitute for estradiol in the synergistic reaction with progesterone to bring about lobulo-alveolar development. In this respect, its estrogenic activity is peculiar, and perhaps, atypical.

The amount of DCA required to produce even slight lobulo-alveolar growth in the rat is much greater than that reported by Mixner and Turner (1942) to produce lobulo-alveolar growth in the mouse. In their experiments, DCA was approximately one-third as potent as progesterone. An exact ratio of DCA to progesterone cannot be determined from the present data. However, it must be pointed out that the amount of progesterone, injected with estrogens, required to produce optimal lobulation is also greater in the rat (Curtiss, 1949, Smith, 1952, in preparation).

The progestin-like activity of DCA in these experiments may be due in part to a conversion of the injected DCA to progesterone, similar to that which has been reported for the human and chimpanzee (Horwitt, *et al.*, 1944), macaque (Zarrow, *et al.*, 1950), rabbit (Hoffman, *et al.*, 1943) and mouse (Hooker and Forbes, 1949). However, this conversion, or other metabolic pathways of DCA have not been demonstrated in the rat. Consequently, we have no hint as to what extent DCA itself or the metabolites of DCA are responsible for the mammotropic effects seen in the present experiments.

Other lines of evidence have implicated the adrenal cortex in mammary growth processes. These are the several reports that adrenalectomy affects mammary growth (Butcher, 1939; Reeder and Leonard, 1944; Trentin and Turner, 1947; Johnston and Smithcors, 1948; Cowie, 1949; Smith, 1951). In the last-mentioned study, lobulo-alveolar growth was unaffected by adrenalectomy, and the reduced duct response to the ovarian steroids and relaxin was restored by administration of DCA, or other corticoids. Other reported effects have varied widely, from slight stimulation to partial suppression of growth, but are not completely unreconcilable.

If the DCA-type compounds play more than a sustaining role for mammary growth in the rat, endogenous production must be enormous, and might even approach a toxic level. Three of nine rats receiving the highest dose of DCA, 10 mg. daily, died during the course of treatment, while rats receiving the propylene glycol alone remained healthy. These results suggest that this is not only a maximal hormonal dose, but also approaches a toxic pharmacological dose for this species.

It might be expected that since DCA resembles progesterone structurally, but does not itself exert the consistent lobulating activity of progesterone, it would interfere with the optimal growth elicited by estradiol and progesterone. The possibility that the doses of DCA were too low, or the dose of progesterone was relatively high needs further scrutiny. However, with the gravimetric ratios used: 1:4, 1:2, and 1:1, no evidence for any inhibitory effect was obtained. Nor was the reverse of this, an additive or synergistic effect, observed.

SUMMARY

Desoxycorticosterone acetate (DCA) alone exerts a duct-stimulating effect on the mammary gland of the immature ovariectomized rat. Alone, or in combination with progesterone, it does not effect any lobulo-alveolar growth. Five mg. of DCA daily, given simultaneously with estradiol, exhibits a slight lobulating action, but doubling the dose of DCA to 10 mg. produces little additional lobulation. No antagonistic or additive effect

of DCA upon the synergistic reaction of estradiol and progesterone in stimulating lobulation could be detected.

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EVIDENCE FOR A DUAL ACTION OF PARATHYROID EXTRACT IN MAINTAINING SERUM CALCIUM AND PHOSPHATE LEVELS[1]

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THE primary mechanism through which the parathyroid glands influence mammalian calcium and phosphate metabolism is still an unsettled problem. The results of past experiments have led to two divergent views regarding the action of the parathyroid hormone: one view maintaining that the principal role of the hormone is the regulation of the osteoblastic and osteoclastic activity of bone tissue, while the other view claims its action to be primarily in controlling the phosphate threshold of the kidney. For a recent review of the work the reader is referred to Greep (1948).

While much of the recent work has been in favor of the kidney threshold theory, evidence has also accumulated suggesting an extra-renal function of the hormone. Considerable work has been reported showing the influence of the parathyroids on bone metabolism both before and after removal of the kidneys (Selye, 1942; Ingalls *et al.*, 1943; and Barnicot, 1948). In addition, Monahan *et al.* (1944) produced a fall in serum calcium in dogs parathyroidectomized after total nephrectomy. Stoerk (1943) prevented a fall in serum calcium levels of rats after total nephrectomy and parathyroidectomy by administration of parathyroid extracts. Similarly, Stewart and Bowen (1951) produced a rise in blood calcium values after nephrectomy in both normal and parathyroidectomized dogs. On the other hand, Tweedy *et al.* (1947, 1950) failed to find any influence of this gland on radio-phosphorus and radio-calcium distribution in nephrectomized rats.

The purpose of this study is to present evidence that parathyroid extract may produce its primary influence on serum phosphate and calcium levels by independent and separate mechanisms.

MATERIALS AHD METHODS

More than 150 male Wistar rats, weighing between 200 and 250 grams were used in these experiments. The parathyroids were removed individually after the method of

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Richter and Birmingham (1941). Nephrectomies were performed in two stages, the left kidney being removed three to four days prior to the right. Since the rat is very resistant to the removal of the parathyroids, possibly due to the presence of accessory tissue, the test for hypoparathyroidism was a drop in serum calcium to below 7 mg.% and the appearance of a partial tetanic response in the animal. Animals not showing the external nervous manifestations were discarded.

Parathyroid extract[3] was administered subcutaneously in a water solution, each injection containing 50 USP units. Four injections were given over a 24 hour period and the animals were killed four to six hours after the last injection. Blood was removed by heart puncture prior to killing. Serum calcium levels were determined by the method of Clark and Collip (1934); serum phosphates as described by LePage (1949).

Except where otherwise indicated, the experimental procedure was as follows: On Day 1, the parathyroids were removed. The left kidney was removed either on the same or the following day. The animals were maintained on stock diet during the period of recuperation. The actual experiment began with the removal of food in the early evening of Day 4. Eighteen hours later, on Day 5, the parathyroidectomized animals which did not show external manifestations of parathyroid deficiency were discarded, and the remaining animals were divided into the various experimental groups. At this time the second kidney was removed and/or the first injection of parathyroid extract was given. Additional injections were given six and twelve hours later and on the morning of Day 6. The experimental procedures were terminated by bleeding the animals on the afternoon of Day 6. Control groups were included in each experiment and were treated identically in respect to food, removal of kidneys, etc.

RESULTS

The animals used in these experiments were divided into ten groups. Those in the first five groups had intact parathyroid glands, while the remaining animals were parathyroidectomized. A brief description of the experimental treatment of each group is given in Table 1, and the results are summarized in Table 2.

TABLE 1

Group	Parathyroid glands intact	Parathyroid glands removed	Kidneys removed		Parathyroid administration
			Left	Both	
1	*				
2	*		*		
3	*		*		*
4	*			*	
5	*			*	*
6†		*	*		
7		*	*		
8		*	*		*
9		*		*	
10		*		*	*

* For time sequence of operations and parathyroid extract administration see Materials and Methods.

† Killed at the time nephrectomy was completed in other animals; these serve as a baseline for further changes in serum and calcium values following complete nephrectomy.

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[3] Lilly's Solution of Parathyroid Extract.

TABLE 2. SERUM PHOSPHATE AND CALCIUM VALUES

Group	Calcium		Phosphate	
	No. of animals	Value ±S.E.	No. of animals	Value ±S.E.
A. *Intact parathyroids*				
1. Stock Animals	9	11.7±0.18	6	7.7±0.12
2. Controls	21	11.2±0.19	6	5.4±0.34
3. PTH*	7	12.1±0.40	7	5.0±0.31
4. Nephx†	11	11.6±0.32	7	14.6±0.61
5. Nephx-PTH	5	11.3±0.42	5	14.2±0.56
B. *Parathyroidectomized*				
6. PTX‡ only	21	5.7±0.19	4	15.3±1.12
7. Prenephx	8	6.7±0.31	7	10.6±0.43
8. PTH	8	10.1±0.57	8	6.3±0.41
9. Nephx	18	5.3±0.23	11	17.7±0.43
10. Nephx-PTH	22	7.8±0.25	16	17.4±0.44

* Injected with parathyroid extract.
† Nephrectomized.
‡ Parathyroidectomized.

COMPARISON OF SERUM CALCIUM VALUES

The average serum calcium value for the normal rat has been found by us to be over 11 mg.% (Group 1). In the animal with intact parathyroids, this value is maintained at least up to 42 hours of starvation (Group 2), and in the totally nephrectomized starved rats is still constant 36 hours after removal of the last kidney (Group 4).

The administration of parathyroid extract for 24 hours to the normal but starved rat produced a questionable rise in the serum calcium value (Group 3). Previous experiments had shown that if this dosage was continued for an additional two days it would, in normal fed rats, raise the serum calcium level to approximately 15 mg.%. As noted by Tweedy *et al.* (1950), no increase was seen in the serum calcium level of nephrectomized rats treated with the hormone for twenty-four hours (Group 5).

In the parathyroidectomized rat it was found necessary to starve the animal in order to produce a marked drop in the serum calcium. Up to six days after removal of the parathyroids, animals maintained on a normal diet usually had a serum calcium value in the low normal range and only rarely did this drop as low as 9 mg.%. However, if food is removed, as done in these experiments on Day 4, the calcium values drop rapidly and 18 hours later will be found to be approximately 6.7 mg.% (Group 7). 36 to 42 hours after removal of the food, the value will have dropped to 5.5 mg.% (Group 6). As seen in Group 9, nephrectomy does not appear to alter significantly this chain of events.

If, after starvation for 18 hours, parathyroid extract was administered to the parathyroid deficient rat, the serum calcium values rose rapidly and

24 hours later were found to be in the low normal range (Group 8). When total nephrectomy was completed prior to injection of the hormone, a rise in serum calcium was still produced though not as marked (Group 10). Thus, a further decrease was not only prevented, but an actual increase in the serum values was observed as shown by comparison of groups 7, 9, and 10.

COMPARISON OF SERUM PHOSPHATE VALUES

The serum phosphate level of normal animals has been found by us to be approximately 7.5 mg.% (Group 1). This value is, however, markedly affected by environmental conditions, particularly diet (Tepperman *et al.*, 1947). Starvation for 36 to 42 hours caused this value to drop to 5.4 mg.% (Group 2). Parathyroidectomy caused a rapid rise in the serum values. After the 18 hour starvation period, the phosphate level of those animals showing external manifestations of parathyroid deficiency had risen to approximately 10.6 (Group 6), and by 36–42 hours was up to 15.3 mg.% (Group 5).

Nephrectomy, in the normal animal, produced a rise in serum phosphate to approximately the same value as the non-nephrectomized parathyroid deficient animal (Group 4). Nephrectomy, however, increased only slightly the already high phosphate level of the parathyroidectomized rat (Group 9).

At the dosage used in these experiments, parathyroid extract treatment had no significant effect on the serum phosphate in the normal animal (Group 3). In the parathyroid deficient animal, the hormone caused a rapid drop in the serum values to a value approaching the normal condition (Group 8). Nephrectomy apparently negated this effect of the hormone entirely (Groups 9 and 10).

DISCUSSION

Since Collip *et al.* (1925) first discovered the influence of the parathyroids on calcium and phosphorus metabolism, it has become well-established that as a result of parathyroid removal the serum calcium level rapidly falls, accompanied by a corresponding increase in the serum phosphate value. Very little emphasis, however, has been placed on the observations, which have been shown before and confirmed by these studies, that the sequence of changes in the serum calcium levels is not influenced by total nephrectomy performed either at or subsequent to the removal of the parathyroids; and that only minor further increases in the phosphate serum values are produced by such removal of the kidneys.

It has also been well established that, in the hypoparathyroid animal in which low serum calcium and high phosphate levels are present, injection of parathyroid extract produces an immediate increase in the excretion of phosphate by the kidneys, accompanied by a lowering of the phosphate and raising of the calcium serum values. Removal of the kidneys prior or during this sequence of events has a marked influence only on the phosphate changes. All effect of the hormone on the phosphate levels seemingly is lost; however, the calcium values *will* continue to rise though the rate is somewhat restricted.

Careful consideration of these data and those presented by the investigators referred to in this work points very strongly to the facts that calcium and phosphate serum values can vary independently of each other; that normal calcium values can be maintained in the presence of phosphate values two and a half to three times their normal value, and that the effect of parathyroid extract on serum calcium of the parathyroid deficient rat is not negated by the removal of the kidneys.

In the light of these facts, one cannot see how any theory of the sequence of events produced by parathyroid extract can be tenable which makes renal excretion of phosphorus the only means by which the hormone can influence calcium metabolism, though indirect influences by means of changes in the phosphate levels very probably occur (Albright and Reifenstein, 1948).

At the present time there appears to be two alternatives: one which would conclude that the hormone exerts its influence directly on only one of these ions, and that the indirect influence on the other is caused, not only by the solubility product of these two ions in the blood, but also by some other extra-renal influence either in the bones or elsewhere; or that the hormone has a dual action, controlling phosphate primarily through its renal effect, and calcium primarily through an extra-renal influence.

Due to the wealth of data showing the influence of the parathyroids on renal excretion of phosphate and to the widely fluctuating serum phosphate levels possible in the presence of a constant calcium value, the latter alternative is considered to be more likely.

SUMMARY

The influence of nephrectomy upon the action of parathyroid extract has been studied in rats. This extract was administered to normal and parathyroid deficient rats which had been nephrectomized in a two-stage operation. Both serum phosphate and serum calcium values were determined. Nephrectomy appeared to nullify all effects of parathryoid extract upon serum phosphate levels. However, the effect of the extract on serum calcium, though somewhat suppressed, was still apparent. Not only did the parathyroid extract prevent a further decrease in serum calcium in the nephrectomized parathyroid-deficient rat, but when 200 USP units were

administered over a 24 hour period following removal of the last kidney, a definite rise in serum calcium was noted. Due to these observations, it is concluded that for its normal function, the parathyroid extract has a direct and independent effect on both calcium and phosphate metabolism.

ADDENDUM

Attention is called to the recent publication by Stewart, G. S. and H. F. Bowen (*Endocrinology 51:* 80. 1952) which appeared since submitting this manuscript. These authors are suggesting that the phosphate excretion factor of the parathyroid gland extract is an artifact, leaving the calcium mobilizing factor the primary function of the parathyroid glands.

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THE EFFECTS OF CORTISONE AND DESOXYCORTICOSTERONE ON THE RENAL TUBULAR REABSORPTION OF PHOSPHATE AND THE EXCRETION OF TITRATABLE ACID AND POTASSIUM IN DOGS[1]

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IT HAS been demonstrated that the renal tubules of dog and man are capable of reabsorbing a fixed and limited quantity of phosphate per unit of time when presented with an excess in the glomerular filtrate (Pitts *et al.*, 1944; Schiess *et al.*, 1948). Since absorptive capacity is independent of glomerular filtration rate over a fairly wide range (Ayer *et al.*, 1947) the renal mechanism which handles phosphate, like that which handles glucose, exhibits a true Tm relationship. More recently, it has been observed, in man, that an increased renal excretion of phosphate follows the administration of cortisone and ACTH. Moreover, negative phosphate balance, depletion of intracellular stores of phosphate and demineralization of bone have been observed in conditions of hyper-adrenocorticism (Albright 1942, Eliel *et al.*, 1951; Ingbar *et al.*, 1951; Pearson *et al.*, 1950; Freeman *et al.*, 1950; Soffer *et al.*, 1950). Although logically inferred, it has not been clearly defined that this increment in phosphate excretion results from an alteration in the renal tubular capacity for phosphate reabsorption consequent upon an excess of adrenal hormone. Accordingly the series of experiments described below was performed to determine the effects of cortisone and desoxycorticosterone on the renal tubular reabsorption of phosphate. Since exchange of hydrogen and potassium ions for sodium bound by urinary buffers plays a significant role in sodium conservation (Berliner, 1951, Pitts 1950), the effects of these steroids on titratable acid and potassium excretion were observed simultaneously. These experiments show that cortisone depresses the renal tubular reabsorptive capacity for phosphate, whereas desoxycorticosterone is without effect. On the other hand, no clear cut effects of either steroid could be demonstrated on the renal mechanisms involved in the excretion of titratable acid and potassium.

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[1] This work was supported by a grant from The American Heart Association.

[2] This work was done during the tenure of a research fellowship of The American Heart Association.

METHODS

In all, 21 experiments were performed on 4 normal and 2 adrenalectomized dogs, both untreated and treated with cortisone or desoxycorticosterone.[3] Prior to the experiments on adrenalectomized dogs, hormone treatment was discontinued for 5–7 days and the animals were maintained with 0.6% salt solution *ad lib.* during this interval. In order to define the maximal tubular capacity for reabsorption of phosphate, the plasma phosphate level and thus the amount presented to the renal tubules were increased by the infusion of neutral sodium phosphate. In some of the experiments the plasma phosphate level was elevated in stepwise fashion; in others a constant infusion of phosphate was maintained. Cortisone, in amounts of 50 to 75 mgm., or desoxycorticosterone, 2 to 5 mgm., was administered intravenously either prior to, or during the course of the experiment. Cortisone acetate was given as a microcrystalline suspension in saline. Dexosycorticosterone was given as the crystalline preparation dissolved in sesame oil and homogenized with saline. Creatinine, which was used to measure the rate of glomerular filtration, was infused with the phosphate in amounts sufficient to maintain optimum plasma levels. Sodium and potassium concentrations of plasma and urine were determined with an internal standard flame photometer. Creatinine was determined colorimetrically by the method of Bonsnes (Bonsnes, 1945). Titratable acid excretion was determined by electrometric titration as described in a previous communication (Pitts *et al.*, 1945). Phosphate was determined on trichloracetic acid filtrates of plasma and diluted urine by the method of Fiske and Subbarow (1925) as modified by Pitts (1933).

RESULTS

Table 1 summarizes the data of three typical experiments performed on three dogs and demonstrates the effect of the administration of cortisone or desoxycorticosterone on the reabsorption of phosphate. In these experiments constant infusions of neutral phosphate were instituted 30 min. prior to the first control period and maintained throughout the experiment. Following two control periods of twenty minutes, the steroid was given and the effect upon phosphate reabsorption, potassium and titratable acidity measured over a period of 60–80 minutes. The experiment at the top of the table illustrates the decrease in tubular reabsorption of phosphate which follows the injection of 50 mg. of cortisone in a normal dog. This occurs within a thirty-minute period following steroid administration. The second experiment shows that desoxycorticosterone has no effect on the renal tubular reabsorption of phosphate. The third experiment demonstrates that cortisone caused a decrease in phosphate reabsorption in an adrenalectomized dog maintained without hormone substitution therapy for six days which was similar to that seen in the normal animal.

Figure 1 shows graphically the average change in phosphate Tm resulting from adrenal steroid injection in four normal dogs. Each box represents the average phosphate reabsorption in mM./min. over a period of 90 to

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[3] We are greatly indebted to the Schering Corporation for the generous supply of desoxycorticosterone used in these experiments.

100 minutes in untreated dogs and following the administration of cortisone or desoxycorticosterone. The average phosphate Tm was calculated by averaging the phosphate reabsorbed in 3–5 different experiments on each dog. From this, it can be seen that cortisone significantly reduced phosphate reabsorption whereas desoxycorticosterone had no such effect.

TABLE 1. THE EFFECTS OF CORTISONE AND DESOXYCORTICOSTERONE ON THE PHOSPHATE REABSORPTION IN TWO NORMAL DOGS (1 AND 2) AND ONE ADRENALECTOMIZED DOG (3)

Urine flow cc./min.	Glomerular filtration rate,	Plasma		Phosphate			Excreted	
		Phosphate mM/L	Potassium mM/L	Filtered	Excreted	Reabsorbed	Titractable acid	Potassium
				mM/min.			mM/min.	
				1 Control				
1.9	75.7	4.26	3.64	.322	.211	.111	.046	.086
2.31	75.5	5.29	3.14	.405	.305	.100	.056	.094
				Cortisone				
3.7	74.8	5.9	3.14	.441	.379	.062	.056	.073
4.8	75.5	6.23	3.0	.470	.430	.040	.075	.089
6.2	81.2	6.48	2.7	.526	.476	.050	.080	.131
				2 Control				
1.2	103	4.07	3.85	.419	.262	.157	.052	.092
1.4	94	4.29	3.85	.403	.266	.137	.050	.072
				Desoxycorticosterone				
3.3	94	4.65	3.85	.435	.304	.131	.107	.063
4.1	102	4.77	3.8	.487	.343	.144	.095	.108
3.8	106	4.80	2.6	.509	.359	.150	.085	.139
				3 Adrenalectomized Control				
2.6	68	5.96	5.5	.405	.203	.202	.159	.063
3.4	69	6.07	5.8	.418	.224	.194	.152	.082
				Cortisone				
2.9	61	5.84	5.5	.356	.228	.128	.110	.128
3.0	61	5.42	5.0	.330	.217	.113	.097	.135
2.7	66	4.95	4.5	.327	.204	.123	.095	.142

TITRATABLE ACID AND POTASSIUM EXCRETION

Figure 2 shows the increase in potassium and titratable acid excretion which occurs as increasing amounts of phosphate are filtered and thus excreted, under normal circumstances and under the influence of cortisone. In the upper part of the figure the sum of potassium and titratable acid, as a measure of sodium conservation, is plotted as a solid line. In the lower part of the figure is shown the increment in sodium excretion which occurs simultaneously with the increased excretion of titratable acid and potassium. In this pair of experiments the amounts of these substances excreted are seen to be somewhat increased following cortisone, especially when smaller amounts of phosphate were filtered. We have presumed this to be related to the decrease in phosphate Tm. The resulting increase in phosphate excretion per unit filtered led to an increase in sodium, potassium and hydrogen ion excretion. However this has not been a consistent finding. In all animals, the general pattern of titratable acid and potassium excretion was not significantly altered by the administration of adrenal steroid. Further, the response of the adrenalectomized salt-maintained animal did not differ from the normal when the animal was presented with a phosphate load. Reference to Table 1 shows that the adrenalectomized animal excretes considerable amounts of titratable acid and potassium following administration of phosphate. Similar results were obtained in 6 experiments on 2 adrenalectomized dogs.

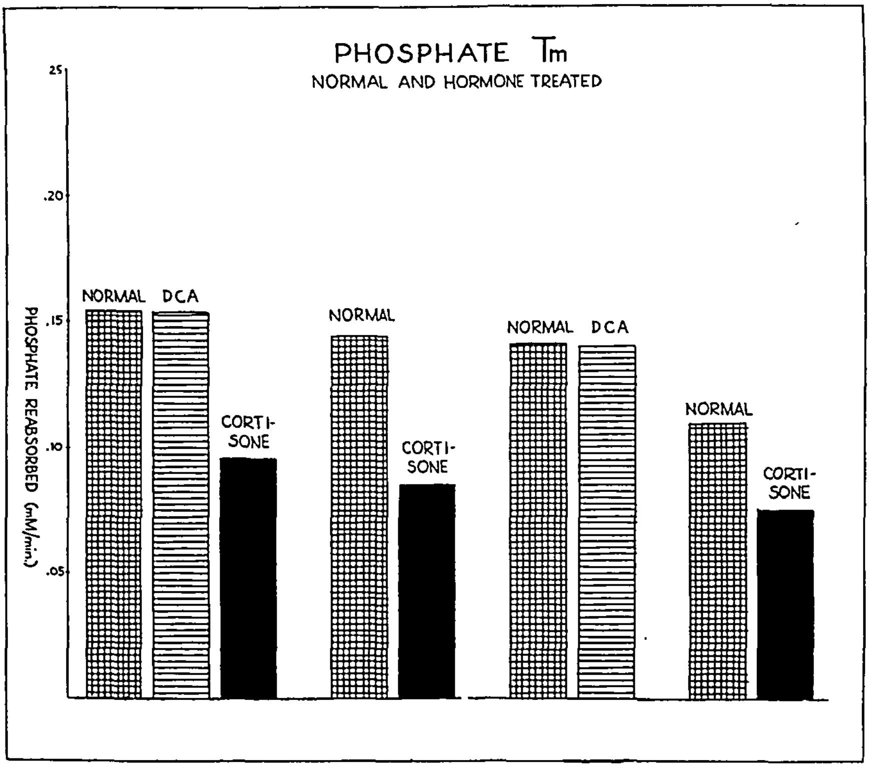

FIG. 1. Effects of cortisone and desoxycorticosterone on the average phosphate reabsorption in four normal dogs.

Figure 3 illustrates further that no specific quantitative differences in titratable acid excretion could be attributed to the influence of adrenal steroid. In this figure is shown the rise in titratable acid excretion which occurs as increased amounts of phosphate are excreted in the normal and adrenalectomized dog, both with and without adrenal hormone. No consistent differences were noted between normal animals and those subjected to adrenalectomy, nor was the excretion of hydrogen ions effected by cortisone or desoxycorticosterone.

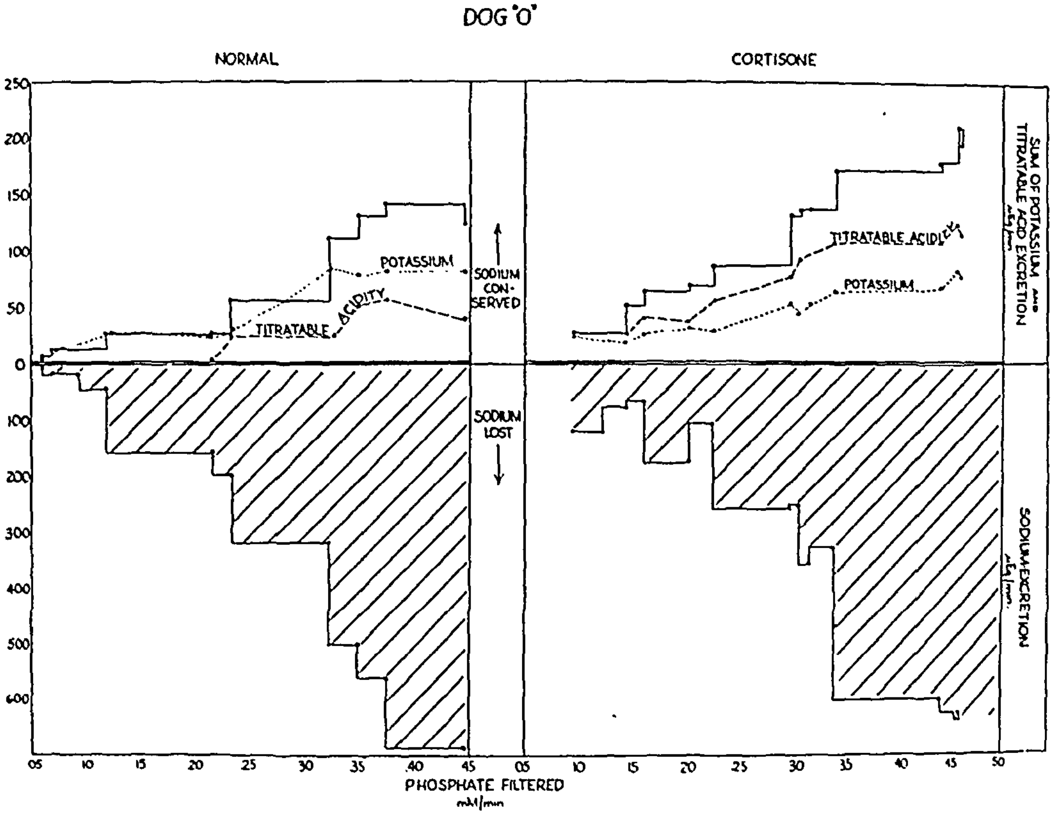

FIG. 2. Sodium, potassium and titratable acid excretion during phosphate loading in a dog with and without cortisone.

DISCUSSION

From the data presented above, it is evident that the depression of phosphate Tm following cortisone administration may be a factor in bringing about the negative phosphate balance observed following the administration of cortisone or ACTH. The increase in the renal excretion of phosphate if continued sufficiently long could account for intracellular depletion of phosphate.

Although other factors may be operative in bringing about the alterations in phosphate metabolism observed in hyperadrenocorticism, these experiments do not elucidate the nature of these mechanisms; nor was any attempt made in these experiments to compare the relative significance of a reduction in phosphate Tm to other factors known to influence phosphate metabolism.

Since the adrenalectomized rat and the Addisonian patient have been reported to show defects in ammonium and titratable acid excretion, it might be expected that the adrenalectomized dog would display a renal deficit in the exchange of hydrogen and potassium ions for sodium (Loeb *et al.*, 1933; Pitts, 1950; Stahl *et al.*, 1936). Such was not found to be the case in our experiments. When presented with a phosphate load, the excretion of titratable acid and potassium did not differ significantly from that seen in normal animals. Further, this response was not modified by cortisone. In fact, no clear cut effects of either cortisone or desoxycorticosterone could be demonstrated upon the excretion of these substances per se: any alterations which did occur appeared to be secondary to those alterations in phosphate excretion which followed cortisone administration. From this, we infer that cortisone does not exert a major influence directly on the renal tubular mechanisms involved with the exchange of hydrogen and potassium for sodium in the dog.

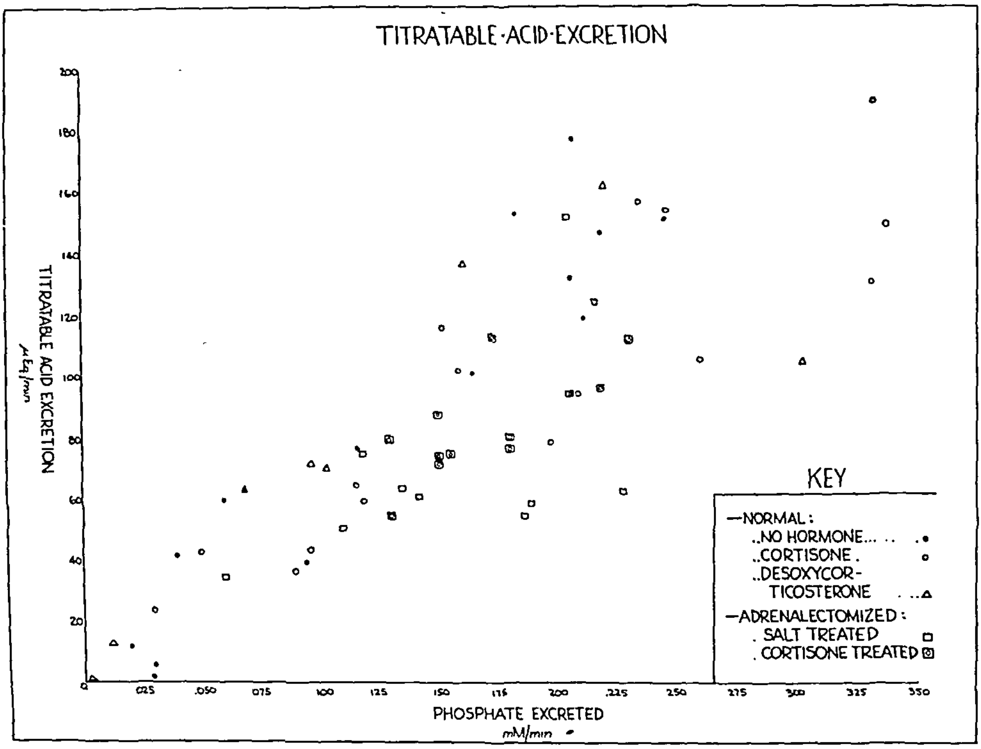

Fig. 3. Titratable acid excretion plotted as a function of phosphate excretion in normal, adrenalectomized and hormone-treated dogs.

CONCLUSIONS

(1) The renal tubular reabsorptive capacity for phosphate is decreased following the administration of cortisone in intact and adrenalectomized dogs. (2) Desoxycorticosterone does not depress the phosphate Tm. (3) The increase in excretion of titratable acid and potassium, which occurs as increasing amounts of phosphate are excreted, is not significantly altered by administration of cortisone or desoxycorticosterone. (4) Adrenalectomized animals maintained on sodium chloride show the same increase in the excretion of titratable acid and potassium following phosphate loading, as that seen in intact animals given a similar phosphate load.

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CYTO-PHYSIOLOGIC ASPECTS OF THYROTROPHIC HORMONE SECRETION IN THE GOITROUS GUINEA PIG*

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THE assignment of cell types to specific hormone secretions in the adenohypophysis continues to thwart proper understanding of the mechanisms underlying thyroid-hypophyseal interplay. Earlier opinion (Severinghaus, 1937) favored the pituitary acidophile as the source of thyroid stimulating hormone (TSH) but recent investigations, based largely on the rat, more clearly implicate the basophiles (Griesbach and Purves, 1945; Brolin, 1945; Goldberg and Chaikoff, 1949; and Halmi, 1950). Purves and Griesbach (1951) moreover, have applied histochemical procedures to the rat adenohypophysis which allegedly discriminate between thyrotrophic and gonadotrophic hormone producing cells routinely classed as basophiles. Despite the apparent relationship between thyroid activation and pituitary basophilia (D'Angelo, 1941; Griesbach, 1951), cellular localization of TSH production in the hypophysis is not satisfactorily established. The general failure to demonstrate increased content of TSH in the rat pituitary after procedures (thyroxine deficiency, cold) which presumably activate the thyrotrophic mechanism clouds interpretation of its morphology (Kuschinsky, 1935; Turner and Cupps, 1940; Griesbach and Purves, 1943). The need for histophysiologic studies of thyroid-pituitary interaction in species other than the rat is consequently urgent. The relatively low content of TSH in the blood and hypophysis of the guinea pig (D'Angelo and Gordon, 1950) suggests a type of thyroid-pituitary balance which may respond markedly to proper endocrine manipulation. Accordingly, a detailed investigation of thyroid and pituitary parameters is being made in this form, one phase of which has already been reported (D'Angelo, Paschkis, Cantarow, Siegel, and Rivero-Fontan, 1951). In the present study, blood and pituitary TSH levels have been determined and correlated with morphologic changes in the thyroid and hypophysis of guinea pigs made hypothyroid with an anti-thyroid agent.

METHOD

A total of 110 normal and experimental guinea pigs were used. Animals received propylthiouracil (0.1%) in the basic ration (Purina Omolene) for periods of 7, 40, and 80

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days, with control animals receiving the basic ration alone. At sacrifice, the thyroid and hypophysis were quickly removed, weighed on a microtorsion balance, and prepared for microscopic study. Pituitaries were divided sagittally, with portions fixed either in Helly's of Champy's fluid. Tissue from the latter were post-osmicated (4–5 days at 37° C.) to demonstrate the Golgi apparatus. Serial sections of paraffin-embedded pituitaries were cut at 2, 3 and 4 micra, and were stained with modified Masson or azan procedures. Thyroid response was evaluated by microhistometric measurement of the acinar cell height (a total of 200 follicular cells per thyroid lobe was measured). Blood for the bioassay study was obtained by cardiac puncture under light ether anesthesia. The sera of animals in respective control and experimental groups were pooled and frozen until ready for assay by the stasis tadpole technic (D'Angelo and Gordon, 1950). The pituitary extract was prepared by a standardized procedure used in the laboratory. Seven anterior lobes (posterior lobe removed by blunt dissection) were homogenized in acidified saline (0.25% glacial acetic acid). The material was extracted twice and, after centrifugation, the clear supernatant fluid was bioassayed for TSH in the tadpoles.[1] Extract volumes were adjusted so that each test animal received an equivalent of 1.4 milligrams of anterior pituitary tissue over a period of 4 injections. For blood assay a total of 0.30 cc. of serum was given in 6 injections.

TABLE 1. BLOOD THYROID-TSH BALANCE AND TSH CONTENT OF THE ANTERIOR PITUITARY IN THE GOITROUS GUINEA PIG

Mean body wgt.-gm. (no. pigs)	Days on drug	M. thyroid wgt.-mg.	M. thyroid cell hgt. (μ)	M. ant. pit.-mg.	Ant. pit. assay		Blood assay	
		100 gm. body wgt.		100 gm. body wgt.	Thy. cell hgt. (μ)	Est. TSH in extract	H-limb increase (mm.)	thyroid cell hgt. (μ)
558±75[1] (7)	0	15.1±1.6	7.1±0.2	1.6±0.07	7.8±0.3	1.6μg.[2] (0.8–2.4)	2.4±0.1[3]	5.1±0.1[3]
435±50 (7)	7	17.2±0.9	7.5±0.3	1.8±0.15	6.9±0.2	0.4μg. (0.2–0.3)	2.4±0.1	5.9±0.1
384±20 (7)	40	26.8±2.6	12.0±0.9	1.9±0.12	8.6±0.3	4.0μg. (2.5–6.0)	1.8±0.1	5.8±0.1
486±50 (7)	78	68.9±9.5	12.2±0.3	2.2±0.09	9.5±0.2	14.0μg. (10–20)	1.2±0.1	5.9±0.2
357±30 (7)	0	16.1±1.0	7.1±0.3	1.8±0.08	7.6±0.2	1.2μg. (0.5–1.6)	2.6±0.2	5.3±0.1

[1] Mean body wgt. at sacrifice ± avg. dev.; in all other columns ± values represent standard error of mean.

[2] TSH content of guinea pig pituitary extracts given in mean microgram equivalents (range) of a previously standardized thyrotropin preparation (D'Angelo, Endocrinology 48: 249, 1951).

[3] Refers to mean hind-limb increase and thyroid cell height in stasis tadpoles receiving guinea pig sera.

RESULTS

The summarized data (Table 1) confirm the earlier finding (D'Angelo *et al.*, 1951) that there is appreciable delay in the onset of morphologic change in the thyroid. Neither thyroid weight nor acinar cell height was changed from normal with seven days treatment of propylthiouracil. The TSH content of pituitary and serum was significantly altered, however. TSH concentration in the adenohypophysis decreased to one-fourth of normal, whereas, blood TSH, not detectable in normal guinea pig serum, rose to levels approximating 0.001 JSU[2]/ml. With continued treatment, elevation of the thyroid cell height, colloid release, and hyperplasia occurred (Fig. 1). The anterior pituitary eventually enlarged, and TSH concentrations in the gland were markedly increased (approximately 280% and 1000% of normal at 40 and 78 days respectively). The elevated blood TSH titers were maintained at a relatively constant level throughout. The decreased hindlimb extension in tadpoles given such sera indicated that the heightened TSH titers were accompanied by simultaneously lowered levels of thyroid hormone. This shift in the thyroid-TSH blood equilibrium follows the inhibition of thyroid hormone formation with goitrogen (D'Angelo and Gordon, 1950).

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[1] Grateful acknowledgment is made to Mrs. Ada Schafer and Mrs. Betsey Brownlee for technical assistance.

The thyrotrophic hormone shifts in blood and hypophysis, as well as the morphologic changes in the thyroid, appeared to be closely dependent upon alterations in the pituitary basophiles. (The detailed changes in hypophysial cell types, as related to disturbances in the thyroid-pituitary axis, will be considered elsewhere; only the major changes which occur with goitrogen will be given here.) The basophilic population, normally concentrated around the area of the stalk along the median, ventral, aspect (bone surface) of the gland showed definite structural changes as early as several days following propylthiouracil treatment. These involved a decrease in the numbers of large, finely granular, deeply staining basophiles and a simultaneous increase in small, coarsely granular cells with hyperchromatic nuclei (Figs. 2 and 3). After prolonged treatment—when TSH concentrations in the gland were appreciably augmented—there was marked hypertrophy and hyperplasia of both light and deeply staining varieties of basophiles, their distribution in the gland becoming widespread (Fig. 4). The dark, irregularly-shaped basophiles were unusually large. These cells were further characterized by a fine cytoplasmic granulation, hypertrophied Golgi networks, and by large nuclei bearing numerous prominent nucleoli (Figs. 5 and 6). Vacuolation was rare, however, and no obvious counterpart to the so-called "thyroidectomy" or "goiter" cell of the rat appeared. There did appear after prolonged treatment, however, certain rather large basophilic cells in the stalk area displaying a coarsely punctated granulation in an otherwise clear chromophobic cytoplasm. Although the acidophiles remained numerous throughout treatment, an appreciable proportion of these cells showed regressive changes. Cytoplasm was visible only as rims or crescents of sparsely granular acidophilic material. Nuclei were pycnotic and the Golgi appratus became inconspicuous. Many acidophiles retained the normal structural pattern, however.

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[2] Junkmann-Schoeller Units.

DISCUSSION

The initial diminution and subsequent marked rise in pituitary TSH content found with chronic goitrogen treatment indicates the necessity of considering the duration factor in cyto-physiologic studies of the adenohypophysis. Failure to consider this may well account for the sharply conflicting reports regarding TSH content of the rat pituitary in thyroxine deficiency (Griesbach and Purves, 1943; Turner and Cupps, 1940; Gordon *et al.*, 1945). The importance of accurate definition of blood TSH levels in conjunction with pituitary examination is equally obvious. It can be safely inferred from the present study that the inhibition of thyroid hormone formation with propylthiouracil elicits augmented release of TSH from the pituitary. With short periods of treatment, thyrotrophic hormone release into the circulation must occur before the mechanism of production is appreciably activated. As thyroid hormone deficiency continues, TSH production eventually surpasses release, so that hormone stores in the pituitary now augment. The relatively constant blood levels of the hormone throughout may mean that delivery of TSH into the circulation, under these conditions of thyroid hormone deficiency, is at a maximal rate. The possibility still exists, however, that increased production of TSH in the pituitary is accompanied by an increased delivery rate of the hormone into the blood with this augmented release of TSH counterbalanced, in turn, by excessive destruction or inactivation in the circulation.

The strong basophilia of the pituitary found at a time when TSH content of blood and hypophysis is high makes it more than probable that the genesis of TSH resides in this cell type. The persistence of the acidophiles in the hypophysis of the goitrous guinea pig, as contrasted with their disappearance in the rat, does not complicate interpretation of the cytologic picture. Neither in numbers, granule content, nor in the behavior of the cytoplasmic organoids, can the acidophiles be correlated with change in TSH stores of the pituitary. The association of this cell type with continued

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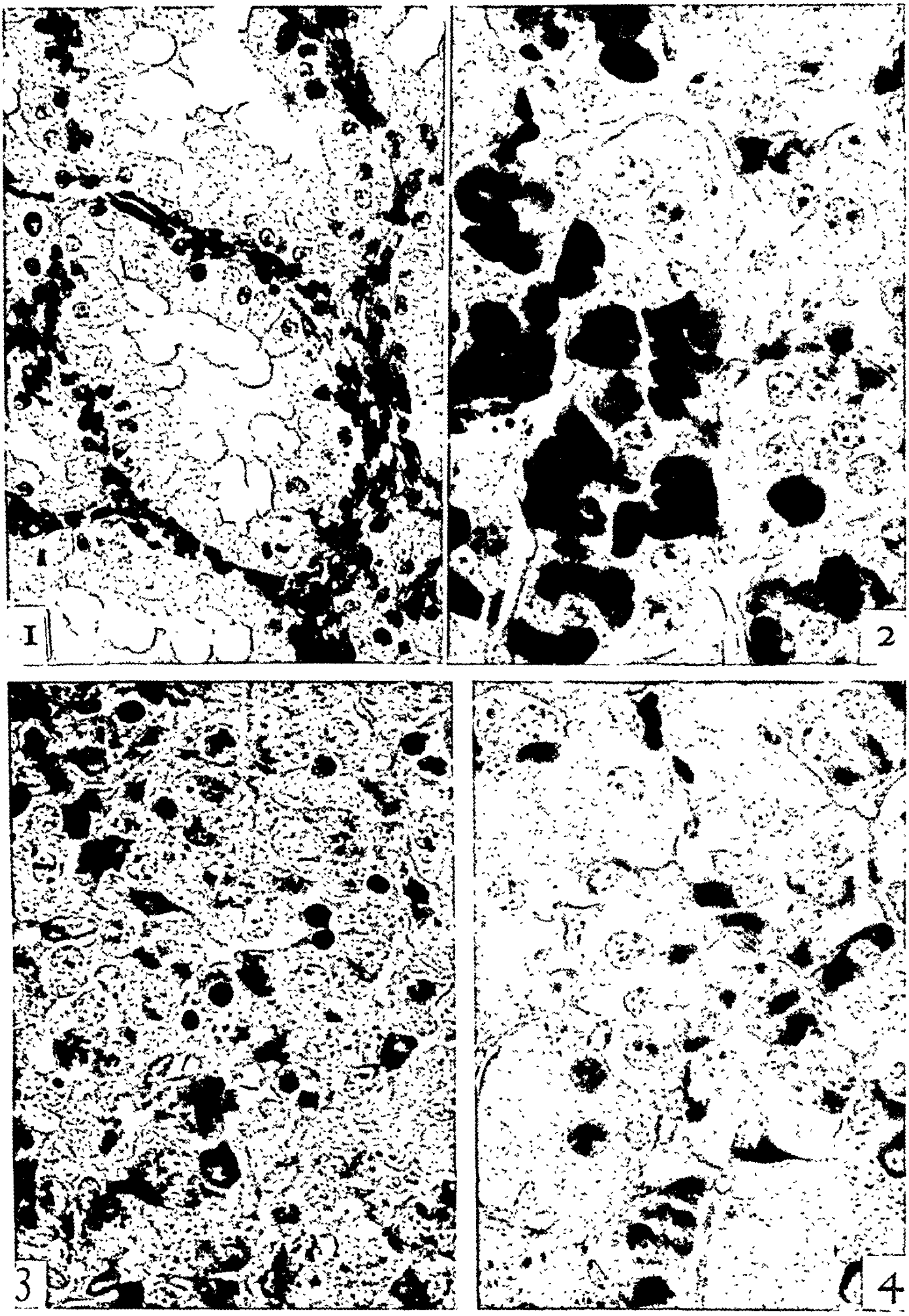
EXPLANATION OF FIGS. 1–4

FIG. 1. The histologic response of the thyroid gland after 78 days treatment with goitrogen (Bouin's Picro-Formol; H. and E., ×350).

FIG. 2. A section through the normal adenohypophysis displaying several large basophiles (upper right) and the more numerous smaller acidophiles (black, elongate elements at left; Helly's, Masson, ×700).

FIG. 3. The widespread degranulation and diminution in size of the chromophiles in the pituitary after 5 days on drug (Helly's, Masson, ×700).

FIG. 4. The adenohypophysis of a guinea pig after 85 days on goitrogen. The basophiles (grey) are hyperplastic; acidophiles (black) are small with rims or crescents of granular cytoplasm (Helly's, Masson, ×700).



Figs. 1–4

EFFECT OF HYPOPHYSECTOMY ON I^{131} METABOLISM IN NORMAL AND THIOURACIL TREATED C_3H MICE

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INTRODUCTION

MANY studies have been made of the effect of hypophysectomy on the radioiodine metabolism of the rat (Leblond, Sue and Chamorro, 1940; Leblond and Sue, 1941; Morton, Perlman, Anderson and Chaikoff, 1942; Cortell and Rawson, 1944; Chaikoff and Taurog, 1949; VanderLaan and Greer, 1950; Ghosh, Woodbury and Sayers, 1951; Randall and Albert, 1951; Randall, Lorenz and Albert, 1951; Albert, Tenney and Lorenz, 1952). Similar studies in the mouse have not been reported as yet.

This paper presents the experimental findings made in a study of some effects of hypophysectomy on I^{131} metabolism in C_3H mice with either normal or hyperplastic thyroid glands.[1] Data are reported on the I^{131} uptake by the thyroid gland, on the percent of thyroid I^{131} in thyroxine, and on the concentration of radioiodide in the thyroid gland compared to that in the serum. It was found that the I^{131} content of the thyroid gland 25 hours after I^{131} injection undergoes an extensive and progressive decrease with increasing time intervals after hypophysectomy. In contrast, while the ratio of radiodide content of the thyroid gland to that of serum decreases after hypophysectomy, it remains elevated above 50.

METHODS AND MATERIALS

The radioiodine studies were made in male C_3H mice between four and five months of age. The mice were supplied by the Animal Production Unit of the National Institutes of Health.

Diet: Two groups of mice were studied. One group was fed from weaning a pelleted stock diet of moderately low iodine content (Wollman, Scow, Wagner, Morris, 1952). After weaning, the other group was fed for three months a meal diet similar in composition to the stock diet but containing a supplement of 2.5 gm. of thiouracil per kilogram. It was then fed the stock diet without supplement for the remainder of the experiment.

Operative procedures: Hypophysectomy was performed through a parapharyngeal approach (Korteweg and Thomas, 1939) when the mice were about four months old. In the sham operation, the hypophysectomy procedure was followed up to and including

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[1] A preliminary report of these results was presented at the New York meeting of the Federated Societies in April 1952. (Wollman and Scow, 1952.)

the removal of the outer table of the occipital bone. Only those hypophysectomized mice are included in this report in which complete extirpation of the gland had been achieved as ascertained by visual examination (9×magnification) of the sella turcica and adjacent tissues. Serial sections of the sella turcica and adjacent bone, stained with hemotoxylin and eosin, were made of the hypophysectomized mice in certain groups as evidence of completeness of the operation. Testes weights were obtained in certain groups of normal and hypophysectomized mice.

I^{131} measurements: The I^{131} in thyroid glands or in extracts of thyroid glands was measured by gamma counting (Wollman, Morris and Green, 1951). The I^{131} in serum was measured by adding 0.05 ml. serum to 0.95 ml. of H_2O containing a mg. of carrier NaI. An aliquot of this solution was dried on a copper planchet and counted with an end window counter. The statistical counting error was less than three per cent by either method. The counting rate of a sample using the beta counter was 150 times that using the gamma counter.

Uptake 25 hours after I^{131} injection: In the 25-hour uptake studies, each mouse received intraperitoneally 10 μc. of carrier free I^{131} [2] and was killed with ether 25 hours later. The thyroid gland, after weighing, was placed in 1 ml. 2 N NaOH and its I^{131} content was determined. The thyroid gland of each mouse fed the thiouracil-free diet was then hydrolyzed on a steam bath, and the thyroxine fraction was isolated by the butanol extraction method of Taurog and Chaikoff (1946) and its I^{131} content determined.

Uptake at 1½ hours after I^{131} injection: One and one-half hours after receiving 10 μc. of I^{131} as described earlier, each mouse was anaesthetized with ether and bled from the heart. The thyroid gland was removed immediately thereafter and was placed in a glass homogenizer with 3 ml. of ice cold 8 per cent trichloroacetic acid solution containing a few mg. of NaI as carrier. It was maintained at a temperature below 5° C. for the remainder of the procedure. The I^{131} content was determined and the tissue was then homogenized in 8 ml. of the acid, centrifuged. and the I^{131} content of the supernatant was measured. The serum I^{131} was determined. Test showed that a negligible fraction of the serum I^{131} was protein bound at this time.

The thyroid iodide/serum iodide concentration ratio: Each mouse in this series was given a subcutaneous injection of 0.5 ml. of freshly prepared solution containing 5 mg. propylthiouracil per ml. of 0.9 per cent NaCl which contained NaOH at a concentration of 0.04 normal. One hour later 10 μc. I^{131} was given intraperitoneally. One to two hours after I^{131} injection the mouse was anaesthetized with ether, a blood sample taken from the heart, and the thyroid gland was immediately removed and weighed. The I^{131} content of thyroid gland and serum were measured.

RESULTS

There was a body weight loss which varied from 4 to 6 grams in hypophysectomized mice (mean body weight at surgery was 30 gm.) during the first 4 to 7 postoperative days after which the weight remained unchanged. The three gram weight loss in the shams was transient; their body weights were back to preoperative values in 7 days. In the mice fed the stock diet, the thyroid weight, which was 2 mg. in normals, was decreased by 26 per cent at 7 days after hypophysectomy and by 39 per cent at 12 days. In mice fed the thiouracil diet the thyroid glands averaged 19 mg. The thyroid weights of mice fed the thiouracil diet decreased by 15 per cent during the 13 days following hypophysectomy. However, the thyroid weights of the sham-operated controls decreased sufficiently so that the thyroid weights in the experimental and control groups never differed significantly. The mean and the standard deviation of the weights of a single testis in mice fed the thiouracil supplement was 86 ± 7 mg. in the shams, whereas in hypophysectomized mice it was 77 ± 7 mg. at six days after hypophysectomy and 40 ± 11 mg. at 13 days.

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[2] The I^{131} used in this investigation was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

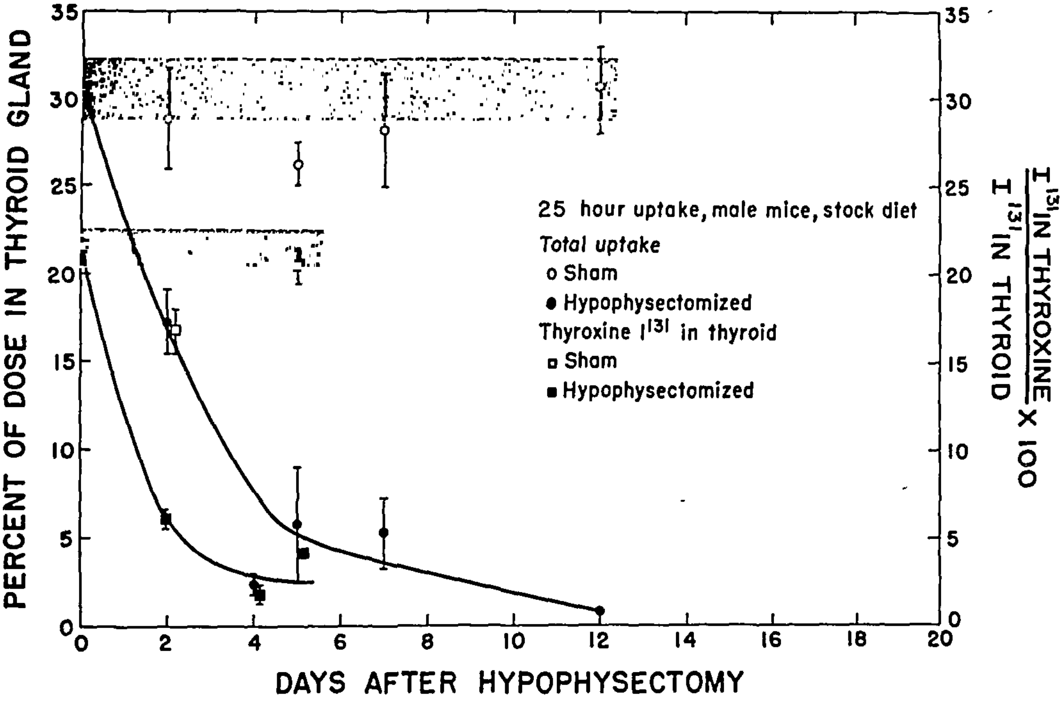

FIG. 1. Effect of hypophysectomy on I^{131} uptake and on percentage of thyroid I^{131} present in thyroxine fraction 25 hours after radioiodide administration in C_3H mice fed the stock diet. The shaded areas indicate the standard error range for the normals; the upper is for I^{131} uptake, the lower for thyroxine fraction. The vertical bar at each point represents the standard error. Four to six mice were used per point. The point for five days after hypophysectomy includes observations on only two mice.

The 25-hour I^{131} uptake at various time intervals after hypophysectomy in mice fed the stock diet is shown in Figure 1. There was a progressive decrease in the uptake of the thyroid glands in hypophysectomized mice during the postoperative period, with the uptake decreasing to 50 per cent of the control value by the second postoperative day.[3] The sham-operative procedure had no significant effect on the uptake even though the left lobe of the thyroid gland had been compressed during the operative procedure.

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[3] In a few data collected on female mice the I^{131} uptake was 30 per cent higher than in corresponding male mice, but there was no significant sex difference in the I^{131} uptake in mice that had been hypophysectomized seven days.

The per cent of thyroid I^{131} in the thyroxine fraction in these mice is also presented in Figure 1. By the second postoperative day, a marked reduction of the per cent of thyroid I^{131} in the thyroxine fraction to one-third of that for the shams was observed in hypophysectomized mice, there being a further decrease thereafter.

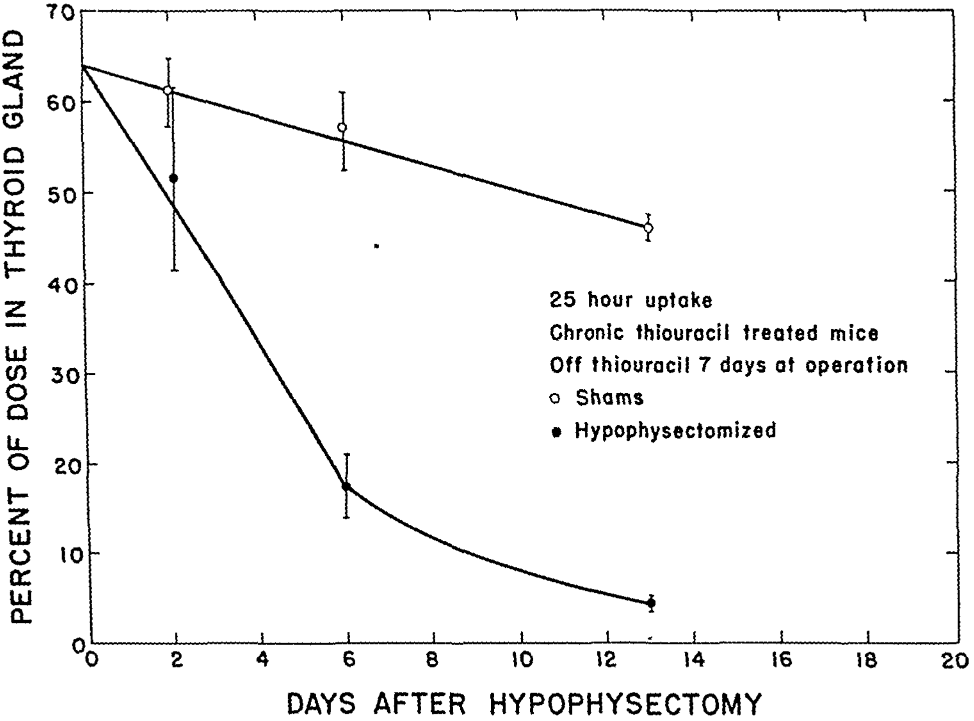

FIG. 2. Effect of hypophysectomy on uptake of radioiodide 25 hours after administration in C_3H mice fed 0.25% thiouracil diet up to seventh preoperative day and then fed the stock diet. Approximately 5 hypophysectomized mice and 6 sham operated mice were studied at each time interval.

The 25-hour I^{131} uptake in hypophysectomized and sham-operated mice fed the thiouracil diet is presented in Figure 2. The mice were fed the stock diet for seven days just prior to operation. Completeness of hypophysectomy was verified by examination of serial sections of the region of the sella turcica in those mice studied at 13 days after hypophysectomy. There was a progressive decrease in the I^{131} uptake following hypophysectomy in these animals, as observed also in those fed the stock diet. The gradual decrease in I^{131} uptake with increase in the postoperative time intervals observed in sham-operated mice probably was the result of the withdrawal of thiouracil. The amount of I^{131} incorporated into the thyroxine fraction in these hypertrophied thyroids was not determined since preliminary studies had shown that it was too small to be measured accurately.

The effect of hypophysectomy on I^{131} uptake 1.5 hours after I^{131} injection in mice fed the thiouracil diet is shown in Table 1. The mice were fed stock diet for 18 days before operation. Completeness of hypophysectomy was verified in each experimental animal by examination of serial sections of the region of the sella turcica. This study was made to check the possibility that the small effect of hypophysectomy on 25-hour uptake at short time intervals after operation in animals fed thiouracil might be due in part to the fact that the maximum uptake in the thyroid glands of the controls occurs only a few hours after I^{131} injection, while the uptake in the hypophysectomized animals increases more slowly, but continues to increase for a much longer time after injection. At this 1.5 hour time interval the total I^{131} in the thyroid glands of the hypophysectomized mice was only 20 per cent that of the controls, the difference being almost twice as great as that observed at 25 hours after I^{131} injection. The concentration of acid-soluble I^{131} in thyroid glands and the concentration of serum I^{131} are approximately 50% higher in the hypophysectomized mice than in the controls. As a consequence, the ratio of the concentration of acid-soluble I^{131} in the thyroid to serum radioiodide concentration was the same for both

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON I^{131} IN THE THYROID GLAND AND SERUM IN C_3H MICE WITH HYPERPLASTIC THYROID GLANDS

		Hypophysectomized	Sham-operated
Number of mice		5	5
Thyroid I^{131}			
Total	counts/sec./100 mg.[1]	109 ±6	495 ±29
TCA-soluble	counts/sec./100 mg.	66.5 ±6	41.2 ± 5
Per cent TCA-soluble		61 ±4	8.3 ± 0.3
Per cent of injected dose			
Total		2.92±0.36	16.6 ± 1.5
TCA-precipitable		1.11±0.12	15.3 ± 1.5
Serum I^{131}	counts/sec./100 mg.[1]	4.11±0.26	2.62± 0.06
$\frac{\text{Thyroid iodide conc.}}{\text{Serum iodide conc.}}$		14.5 ±1.2	16.2 ± 1.9

[1] All counting rates reported as if measured by gamma counting. The serum data were obtained by beta counting, and were converted to the equivalent gamma counting data using an experimentally determined conversion factor.

Mice fed diet containing thiouracil for three months, fed stock diet 18 days, and then operated on. Studies were made on sixth postoperative day. Samples were taken one and one-half hours after I^{131} administration.

groups. Acid-soluble I^{131} amounted to 60% of the total I^{131} in the thyroids of the hypophysectomized mice, but only 8% in the controls.[4] The amount of organic bound I^{131} in the thyroids of hypophysectomized mice was much less than in those of the controls.

The effect of hypophysectomy on the thyroid iodide/serum iodide concentration ratio in mice fed thiouracil is presented in Table 2. A statistically significant though small depression of this ratio as the result of hypophysectomy was observed only at thirteen days after operation, the latest time interval studied. The hypophysectomies in this last group, studied 13 days after operation, were shown to be complete by examination of serial sections of the region of the sella turcica. Two and one-half mg. of propylthiouracil was shown to be effective in blocking protein binding of radioiodide by the thyroid gland for at least five hours in these mice. The values of the ratios in the sham-operated mice were abnormally low, since mice fed either a goitrogen-free diet or thiouracil in this laboratory ordinarily have ratios of over 200. The low values observed may have been due to a somewhat higher iodide concentration in the particular batch of stock diet fed the mice in the time interval during which these studies were made.

TABLE 2. EFFECT OF HYPOPHYSECTOMY ON THYROID IODINE/SERUM IODIDE CONCENTRATION RATIO[1] IN C_3H MICE WITH HYPERPLASTIC THYROID GLANDS[2]

Days after operation	Concentration ratio	
	Hypophysectomized	Sham-operated
2	75 ± 8 (5)[3]	96 ± 4 (7)
6	90 ± 10 (6)	91 ± 9 (7)
13	67 ± 6 (6)	108 ± 9 (6)

[1] In order to prevent organic binding of radioiodide, each mouse was given a single subcutaneous injection of a solution containing 2.5 mg. of propylthiouracil one hour prior to injection of 10 μc of I^{131}. Samples were taken one to two hours after the I^{131} injection.

[2] Fed thiouracil diet three months, hypophysectomized after feeding stock diet seven days.

[3] Mean ± standard error; parenthesis encloses the number of animals examined.

DISCUSSION

The effects of hypophysectomy on the I^{131} metabolism in the mouse appear qualitatively similar to those in the rat. The results, however, suggest some interpretations of the effects of hypophysectomy which are different from interpretations in papers on the rat.

The I^{131} content of the thyroid glands twenty-five hours after I^{131} administration decreased progressively with increasing time intervals after hypophysectomy in mice fed either the stock diet or the thiouracil-containing diet. Although not shown in figure 1 the logarithm of the uptake of the thyroid glands decreased linearly with the time after hypophysectomy in the mice fed the stock diet. This indicates a constant fractional rate of decrease of the 25-hour uptake with time after hypophysectomy. This interpretation may be contrasted with that suggested by Randall and Albert (1951) who concluded that the thyroid activity in the rat reached a minimum of 10% of normal by six days after hypophysectomy (using maximum uptake of injected I^{131} as a measure of thyroid activity). The interpretation of these data on the rat as well as the present 25-hour uptake data is complicated by the fact that the maximum uptake reflects not only thyroid function but also kidney function, and in particular the fact that radioiodide excretion by the kidney is grossly decreased in the hypophysectomized animal (Albert, Tenney and Lorenz, 1952). This would tend to make injected I^{131} available to the thyroid gland in the hypophysectomized animal for a longer time than in the normal, and tend to decrease the apparent loss of thyroid activity if a long time interval between injection and thyroid I^{131} measurement is selected. For a reliable measure of thyroid activity it is important to study a characteristic which is relatively independent of kidney activity such as thyroid clearance or uptake at times so soon after I^{131} administration that only a small fraction of administered I^{131} could be excreted by the kidney even in the normal animals.

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[4] Albert and Lorenz (1951) have obtained similar results in the rat.

It is important to ascertain which of the factors involved in the accumulation of I^{131} by the thyroid gland are grossly affected after hypophysectomy and responsible for the observed decreased accumulation. The preliminary observations on organic bound I^{131} six days after hypophysectomy in the mice with hyperplastic thyroid glands indicates a marked decrease in organic bound I^{131} in the hypophysectomized mice. This correlates well with the prompt decrease in the proportion of thyroid I^{131} found in the thyroxine fraction in mice fed the stock diet and previously reported for the rat (Morton, Perlman, Anderson and Chaikoff, 1942). Both organic binding of I^{131} and formation of thyroxine are presumably linked to oxidative processes in the cell.

The decrease in the organic bound I^{131} in the thyroid glands of the hypophysectomized mice with hyperplastic glands does not appear to be due to a lack of radioiodide in the gland since the acid-soluble I^{131} was actually higher in their glands than in those of the control mice. If the relationships at 1.5 hours after I^{131} injection are representative of those at earlier times it would suggest that the process of organic binding of I^{131} was grossly impaired in hypophysectomized mice.

The values reported for thyroid iodide/serum iodide concentration ratios of mice hypophysectomized after chronic feeding of thiouracil are almost always above 50. Many additional measurements of this ratio in mice hypophysectomized after feeding a thiouracil-free diet since weaning, to be reported elsewhere, have repeatedly confirmed the above observations. These results in the mouse differ markedly from those in the hypophysectomized rat reported by Greer (1949) who found that the ratio approaches approximately 7, and by VanderLaan and Greer (1950) who found that the ratio frequently approaches 1. The relative insensitivity of this ratio to hypophysectomy in the mouse makes it possible to recognize the importance of the binding process as an important factor in the decreased accumulation of I^{131} in hypophysectomized mice.

SUMMARY

The I^{131} content of the thyroid glands twenty-five hours after I^{131} administration in male C_3H mice with normal or hyperplastic thyroid glands decreased progressively with increasing time after hypophysectomy. The proportion of the thyroid I^{131} in the thyroxine fraction also decreased rapidly after hypophysectomy in mice fed the stock diet. The thyroid iodide/serum iodide concentration ratio in the mice with hyperplastic thyroids exhibited a decrease during the 13 days after hypophysectomy but remained elevated above 50.

The decreased accumulation of bound I^{131} in the thyroid glands of these hypophysectomized mice did not appear to be due to a decrease in thyroid radioiodide concentration.

Acknowledgement

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MECHANISMS OF PARTIAL BLOCKADE OF THE STRESS RESPONSE IN RATS BY DIBENAMINE ANALOGUES[1]

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INTRODUCTION

IT IS generally agreed, following the pioneer work of Long and Fry (1945) and Vogt (1945), that physiological dosages of epinephrine will activate the release of adrenocorticotrophin (ACTH) from the adenohypophysis. However, there is considerably less accord as to the mechanism by which epinephrine exerts this effect. Long and his colleagues (Gershberg *et al.*, 1950; McDermott *et al.*, 1950) have presented strong evidence, confirmed by Fortier (1951 b), that injected epinephrine may act directly on the adenohypophysis. Hume and collaborators (Recant *et al.*, 1950) suggest that epinephrine activates the anterior hypothalamus to liberate a humoral substance which, in turn, stimulates the hypophysis. Sayers (1950, 1951) considers that epinephrine increases tissue utilization of cortical steroids and that their depletion from the blood "fires" the ACTH release mechanism. Porter (1952) has recently reported direct evidence that epinephrine activates the posterior hypothalamus and that lesions in this region prevent stress-induced discharge of ACTH. DeGroot (1952) also favors the hypothalamus as the primary site of epinephrine action, for he found that epinephrine would not stimulate lymphopenia in mice with their hypophyseal portal systems damaged.

The hypothesis that reflex secretion of endogenous epinephrine is essential in the natural mechanism by which stress activates ACTH release is confronted by similar lack of agreement. Gordon (1950 a, b) reported a normal response to "alarming" stimuli in rats with demedullated adrenal glands. This was denied by Gershberg *et al.* (1950) and Dury (1950). The controversy as to whether epinephrine is a specific link in the pituitary-adrenal mechanism or whether it acts like other non-specific stresses, is treated in the discussions of recent symposia reviews by Pincus (1950), Sayers (1951), Harris (1952) and Long (1952). Long's group has proposed a

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dual mechanism in which systemic epinephrine is responsible for an initial *autonomic* phase of pituitary stimulation but not for the later *metabolic* phase.

A barrier to the acceptance of a specific role for intrinsic epinephrine is the almost universal failure of attempts with adrenergic blocking agents to interfere with ACTH release. Partial blockades of the mechanism by ergotamine (Ronzoni and Reichlin, 1950) and by Dibenamine (Seifter *et al.*, 1949; Paschkis *et al.*, 1950; Ronzoni and Reichlin, 1950) have been reported. Not only was the degree of blockade slight but there was no attempt made to test whether even the partial effect was actually due to adrenergic blocking properties of the drugs. This latter consideration assumes importance in view of results to be presented below. Moreover, in the hands of other investigators, Dibenamine (Tepperman and Bogardus, 1948; Fortier, 1951 a), ergotamine (Tepperman and Tepperman, 1950; Gershberg *et al.*, 1950) and dihydroergocornine (August and Gubner, 1949) have been completely ineffective in interrupting the stress response. Whereas these negative results are inconclusive, they have failed to lend support for a significant role of endogenous epinephrine in the ACTH release mechanism.

In 1950, Sawyer *et al.*, reported that a new adrenergic blocking agent SKF-501 (N [9-Fluorenyl]-N-ethyl-β-chlorethylamine hydrochloride) was more highly effective than Dibenamine in blocking the neurogenic stimulus for LH (ovulating hormone, gonadotrophin) release from the adenohypophysis of rabbits and rats. SKF-501 has been shown to have 3-10 times as potent adrenergic blocking properties as Dibenamine (Fellows, 1949; Sawyer *et al.*, 1950). In adrenergic-blocking dosages, SKF-501 prevented ovulation in 80 per cent of the rabbits and rats tested. The mechanism of blockade was apparently anti-adrenergic activity since the effectiveness of SKF-501 was not shared by 2-dibenzylaminoethanol (D-ol), a Dibenamine derivative affecting the central nervous system but lacking adrenergic-blocking capacity. It seemed desirable, therefore, to examine the effects of both SKF-501 and D-ol on the stress response. The present study reveals that SKF-501, but not D-ol, largely blocks the stress-response to injected epinephrine in rats as evidenced by effects on the eosinophil count. Interestingly enough, both SKF-501 and D-ol prevent the autonomic phase in response to such non-specific stresses as cold and formalin injection. The results are interpreted as indicating that D-ol interrupts the reflex arc for neurogenic secretion of endogenous epinephrine in response to non-specific stress, and that SKF-501 may function in this manner as well as having the capacity to block the action of epinephrine at its effector site. The results are consistent with the sequence proposed in the dual hypothesis of Long *et al.* (*op. cit.*). Part of the present results, with tentative interpretations somewhat at variance with the present ones, have appeared in abstract form (Sawyer and Parkerson, 1951; Sawyer, 1952).

MATERIALS AND METHODS

The experiments fall into two principal groups: those conducted in North Carolina (Series A) and those in California (Series B). In Series A, male rats of the inbred Vanderbilt (Osborne-Mendel) strain, ranging in weight from 220–360 grams, were used in the hematological study. The rat room was air-conditioned at a summer temperature of about 27° C. The animals were kept in wire mesh cages, not more than three to a cage, and fed *ad lib* on Purina Dog Chow. Lighting conditions were controlled by a time switch to give 14 hours of light per day (Everett, 1948). In Series B, Wistar rats ranging in weight from 150–260 gm. were employed. Lighting and temperature conditions were not rigidly controlled but on the day of each experiment, care was taken that the rats not be disturbed prior to the administration of the controlled stressing stimulus. Other conditions were similar to those in Series A. In both series, the stressing stimulus was applied routinely between 9–10 A.M.

The method employed to assess ACTH release was the eosinophilic response of Thorn *et al.* (1948), and direct eosinophil counts were made with a modification of Randolph's (1944) phloxine-propylene-glycol diluent. A small blood sample was collected from the tail vein into an oxalated depression in a paraffin block. Within a few minutes, in Series A, three WBC pipettes were filled to the 0.1 mark and diluted to the 1.1 mark. They were shaken for about 30 seconds and set aside for at least 15 minutes for staining. After shaking again at a fairly rapid rate for 2 minutes in a mechanical shaker, each of two pipettes was used to fill 4 Spencer Bright-Line chambers, with a 30-second period of shaking between chambers. If the 36-square counts for each of the two pipettes checked within 15%, the third pipette was not used; if not, the average of all three was taken. Most of the Series A eosinophil counts for control rats ranged between 30–80 cells per cubic mm., and repeated counts on individual animals, with a few days interval between bleedings, gave very similar values. These counts are lower than most of those reported heretofore, indicating that strain differences in rat eosinophil levels may exist. Most of the Series B Wistar rat eosinophil counts ranged between 100–300 cells per cubic mm. Seventy-two squares filled from 2 WBC pipettes were counted in the eosinophil determinations and rats were not used whose pre-stimulation level was less than 70/cu. mm.

Three types of stimuli for ACTH release were employed: epinephrine, cold stress, and formalin. Epinephrine (Parke-Davis Adrenalin, 1/1000–1/5000) was injected subcutaneously at a dosage of 0.2 mg./kg. Ten per cent formalin was injected subcutaneously at a constant dosage of 0.3 ml. As a cold stress stimulus, rats were kept for one hour in a refrigerator room at a temperature of 8–10° C., nearly 20° C. less than the rat room temperature. In the cold stress groups, blood samples were taken at 0 and 4 hours, so the stimulus includes handling and bleeding as well as the temperature changes. Control blood samples in the Series A epinephrine group were taken a few days before or after the experimental 4-hour sample, so that the effects of epinephrine were not complicated by additional stress. The Series B eosinophil counts assayed blood samples taken at 0, 1 and 4 hours in both the epinephrine and formalin series, so the handling and bleeding element in the stress stimulus was considerable. Routinely there was a 4-day recovery period between consecutive stress experiments on a given animal, and more than a week was allowed for recovery from SKF-501 or D-ol injection.

Sodium pentobarbital (Nembutal, Abbott), which was employed intraperitoneally in preliminary experiments in Series A, was in the form of the veterinary preparation in an alcohol-propylene-glycol-water vehicle or a 2 per cent solution in Ringer-Locke. The latter solution, which was made up just before use, was warmed to body temperature and injected intraperitoneally at a dosage of 30–50 mg./kg.

The adrenergic blocking agent, SKF-501, was dissolved in a propylene-glycol vehicle at a concentration of 50 mg./ml.; this was diluted for use to 5 mg./ml. with Ringer-Locke solution. Intravenous injections were performed into the lesser saphenous vein below the ankle as described earlier (Sawyer, Everett and Markee, 1949). The dosage adopted for use, 10 mg./kg., is above the MLD: in the present experiments 2 rats receiving this dose died during the following night. This dosage level was chosen because of its demonstrated ability in the rat and the rabbit to block the stimulus for release of pituitary LH without interfering with the release mechanism itself or with the action of hormone on its target organ (Sawyer *et al.*, 1950). Dibenzylaminoethanol was injected intravenously at a concentration of 10 mg./ml. in saline, and the dosage of 15–20 mg./kg. usually induced immediate convulsive muscular activity which lasted about one minute. Thereafter, the rats' motor activity appeared to be depressed.

RESULTS

Stress responses induced by Nembutal, SKF-501, and D-ol. Our first experiments were an unsuccessful attempt, with Nembutal anesthesia, to stabilize the eosinophil count against depression from handling and bleeding. It was hoped that even intravenous injections of SKF-501 might be made under anesthesia without inducing an alarm reaction. Recant *et al.* (1950) and Ronzoni (1950) had reported such a stabilizing effect of the anesthetic. As summarized in Figure 1, our results were completely negative in this respect. Nembutal itself, in confirmation of Ludewig and Chanutin (1947), acted as a stress stimulus leading to a 50% reduction in the eosinophil count after 4 hours. It appeared to make little difference whether the Nembutal was in alcohol-propylene-glycol-water or in Ringer-Locke buffered with sodium carbonate, at room temperature or warmed. Nor did "training" the rats with twice-daily mock injections for a week lessen the stress response.

In spite of the fact that its central excitatory effects are much less severe than those induced by Dibenamine (Sawyer *et al.* 1950), intravenous injection of 10 mg./kg. SKF-501 was followed by a marked reduction in circulating eosinophils (Fig. 1). When the SKF-501 injection was preceded by intraperitoneal Nembutal the decrease was somewhat less, but the number of cases was too small to make the difference significant. Even if it were significant and if SKF-501 were completely ineffective in blocking ACTH release, a 60 per cent decrease in eosinophils is already so great that one could not expect much further reduction in response to cold stress or epinephrine. Inasmuch as the adrenergic blocking capacity of β-chloroethylamine derivatives lasts for days (Nickerson, 1949) we next tested whether the eosinophil count would return to normal by 24 hours after injection of SKF-501. This was observed to be true (Fig. 1), and the finding allowed us to examine the drug's capacity to block the eosinopenic response to stress at that time. Marked eosinopenia was also induced by injection of D-ol, followed within 24 hours by a return to nearly normal levels (Fig. 1). Later results with Wistar rats (Series B) revealed eosinophil counts characteristically higher than normal, 24 hours after injection of either SKF-501 or D-ol.

To demonstrate that adrenergic blockade by SKF-501 does indeed persist for 24 hours after treatment, we injected 3 rats with 5 mg./kg.

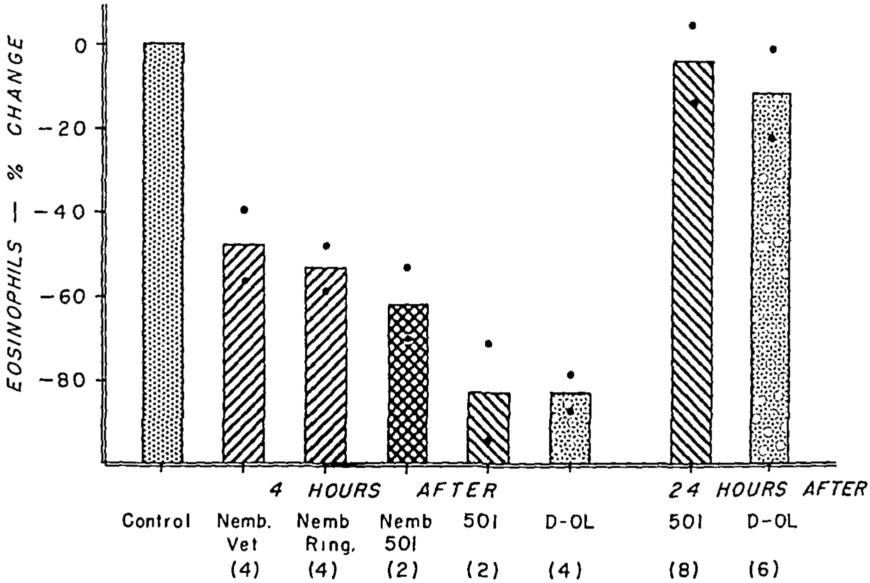

FIG. 1. Results of preliminary experiments in Series A. The effects of Nembutal, SKF-501, and D-ol on circulating eosinophils are expressed as percentages of the 0-hour counts. The figures in parentheses represent the number of rats employed in each group. Each set of dots limits the standard error of the mean.

SKF-501 (only one-half our usual dose) and followed at 24 hours with 0.2 mg./kg. intravenous epinephrine. This dosage of epinephrine was found in preliminary experiments to be in the realm of twice the LD_{100}, and in the present series it killed 2 control rats and 3 rats injected 24 hours earlier with 15 mg./kg. D-ol, in less than 5 minutes, all with symptoms of pulmonary edema. The 3 SKF-501-epinephrine-treated rats showed no such symptoms, and all survived. Thus SKF-501, at a dosage only half as great as that used in our ACTH experiments, protects at 24 hours against a twice-lethal dosage of epinephrine, whereas D-ol, at full dosage, lacks the capacity to block epinephrine effects.

Effects of SKF-501 and dibenzylaminoethanol on the 4-hour eosinophil responses to epinephrine and to cold stress. The results are summarized in Figure 2. Each of the columns represents data on at least 8 rats except the last one in which only 2 animals were employed. However, only 17 rats were used in this whole series, and as far as possible the same rats were subjected to a given stressful stimulus and attempted blockade of that stimulus. It is apparent that either subcutaneous epinephrine or exposure to cold stimulates a marked decrease in the number of circulating eosinophils, about a 70 per cent depression in either category. The eosinopenia stimulated at 24 hours after SKF-501 injection, though still considerable in the cold-stressed or epinephrine-treated rats, is significantly reduced.

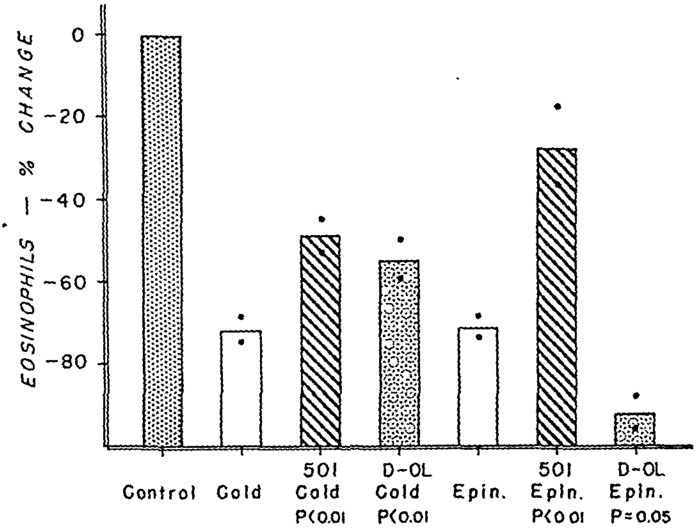

FIG. 2. Effects of "blocking" agents on the 4-hour eosinophil response to cold stress and to epinephrine (Series A). The P values for the differences between stressed and stressed-"blocked" animals were calculated from eosinophil-count data, not from the percentage-change columns shown in the figure.

$P < 0.01$ in either case between the eosinophil levels of SKF-501-treated and non-treated groups. The failure to respond 24 hours after SKF-501 is probably not attributable to the initial non-specific stress of SKF-501 itself, for 4 controls each gave a normal eosinophil response to epinephrine or cold stress 24 hours after epinephrine or cold stress, respectively. Interestingly enough, however, D-ol "blocked" the response to cold stress about as effectively as SKF-501 without preventing epinephrine-induced eosinopenia at all. The degree of eosinopenia in the D-ol-epinephrine group was, if anything, greater than in the controls lacking D-ol.

Effects of SKF-501 and dibenzylaminoethanol on the 1-hour and 4-hour eosinophil responses to epinephrine and to formalin. These experiments were undertaken after the appearance of Long's dual hypothesis (McDermott *et al., op cit.*) to determine whether the autonomic phase of pituitary stimulation might be prevented by adrenergic blockade. The results are summarized in Figure 3. Pre-treatment with SKF-501 but not with D-ol completely eliminated the eosinopenia at 1 hour after epinephrine. The rats pretreated with D-ol again showed a response to epinephrine, perhaps more marked than the controls. The 4-hour results confirm the earlier findings with epinephrine, except that the SKF-501 column reflects the added stress of handling and bleeding at 0-hour and 1-hour collectively.

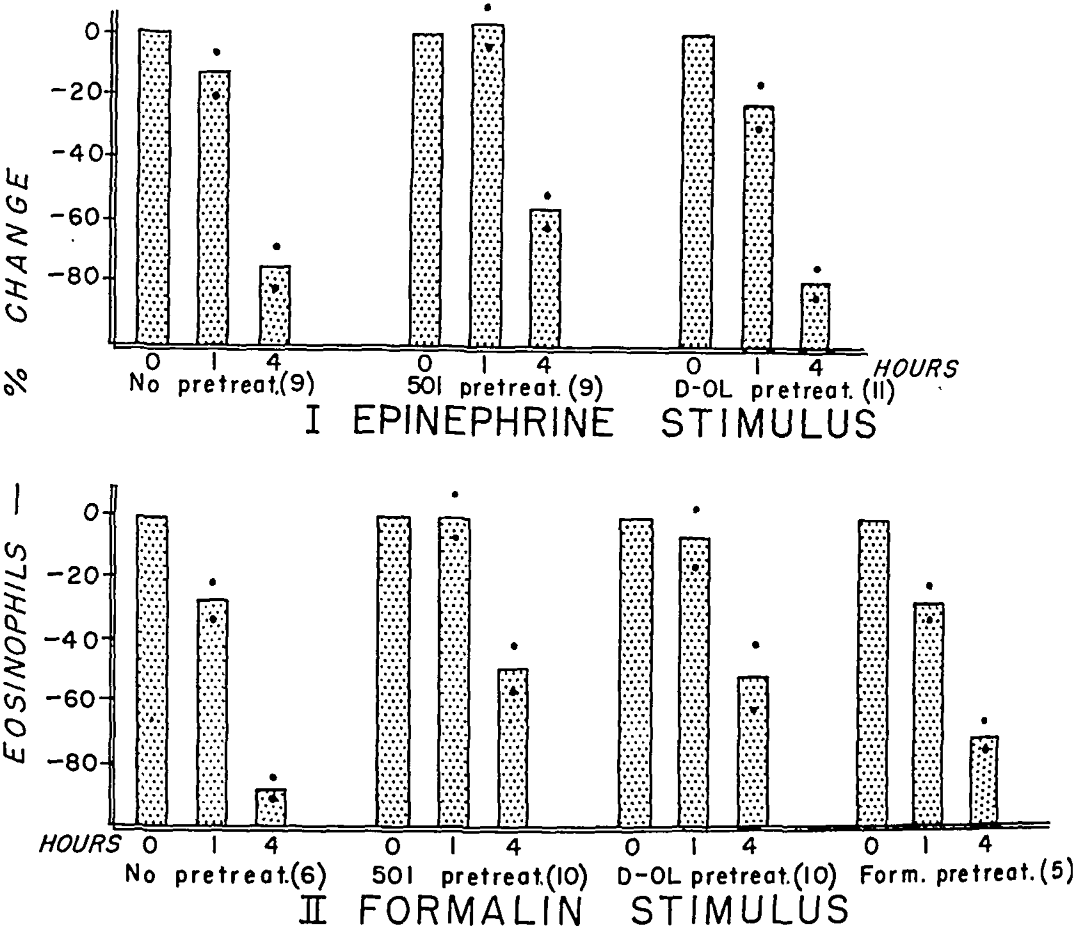

FIG. 3. Effects of pretreatment with SKF-501 and D-ol on the 1-hour and 4-hour eosinophil responses to epinephrine and to formalin (Series B). In group I, epinephrine was injected subcutaneously, 24 hours after treatment with SKF-501 or D-ol. In group II, formalin was injected subcutaneously 24 hours after treatment with SKF-501, D-ol or formalin itself. Figures in parentheses represent numbers of rats employed in each group.

Formalin (Fig. 3) induced an eosinopenia at 1 hour at least as great as that stimulated by epinephrine and the effect was not lessened by formalin stress on the preceding day. However, both SKF-501 and D-ol appeared to prevent this 1-hour response to formalin and to diminish the 4-hour response in the same degree as they had lessened the response to cold.

DISCUSSION

It seems clear from the present results that β-chloroethylamine adrenergic blocking agents may partially prevent stress-induced eosinopenia by more than one mechanism. SKF-501 and dibenzylaminoethanol exert similar degrees of "blockade" against formalin or cold stress but differential effects against epinephrine. The blockade of formalin and cold stress by D-ol must be exerted by some means other than adrenolytic action. The fact that epinephrine is effective in stimulating a normal eosinopenia in spite of D-ol indicates that the final stages in the eosinopenic mechanism are not disturbed by chloroethylamine derivatives. Therefore, the failure of cold or formalin to induce a full-fledged eosinopenia after D-ol or SKF-501 cannot be attributed to the inability of the effector end of the eosinopenic mechanism to respond. Presumably the adrenal cortex and the pituitary ACTH release mechanism are intact.

While lacking the capacity to counteract epinephrine, D-ol blocks the autonomic phase of eosinopenia in response to cold or formalin. It seems most likely that it does so by interfering in some way with the stimulus for release of endogenous epinephrine. In this capacity its action may be analogous to those of barbiturates which were reported by Sayers and Sayers (1946) and Gellhorn (1949) to interfere with adrenal ascorbic acid depletion and lymphopenia, respectively, in the rat after generalized stress but not after injection of epinephrine. D-ol does, in fact, appear to be a depressant after a brief period of excitation of the central nervous system.

SKF-501 exerts a similar though less marked depressant effect, and its primary action against formalin and cold stress may be the same as that exerted by D-ol. However, SKF-501 has, in addition, very potent adrenolytic properties and these are credited with its blockade of epinephrine-induced eosinopenia. There is no evidence that epinephrine was responsible for any of the 4-hour eosinopenia encountered after cold, formalin or epinephrine injection in SKF-501-treated animals. Where non-specific stress was least (i.e. epinephrine injection without bleeding), the 4-hour eosinopenia was least evident.

It is significant that SKF-501 does not prevent the 4-hour eosinopenia from cold or formalin any more effectively than does D-ol. This must mean that under either SKF-501 or D-ol, neither cold nor formalin stimulates a systemic discharge of epinephrine and that the eosinopenia which is achieved is effected via ACTH release from another mechanism, e.g., the "metabolic" phase. The results allow one to make a rough calculation of the relative part played by epinephrine in both autonomic and metabolic phases of eosinopenia. With epinephrine eliminated via blockade, the non-specific metabolic effects induced about zero % and 50% eosinopenia at 1 and 4 hours, respectively, whereas the combined autonomic and meta-

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NOTES AND COMMENTS

ADSORPTION OF OXYTOCIN UPON OXIDIZED CELLULOSE

In the course of using oxidized cellulose for selective adsorption and elution of adrenocorticotrophic hormone, by the method of Astwood *et al.* (1951), it was noted that the oxytocic contaminant of the ACTH was removed along with adrenocorticotrophic activity, but unlike the corticotrophic factor it was not appreciably eluted with 0.1 N HCl (1952). Dumm (1952) has shown that the antidiuretic factor can be quantitatively adsorbed upon oxycellulose and completely eluted therefrom by methods similar to that utilized for ACTH (1).

The present communication deals with the attempts to selectively adsorb and elute the oxytocic principle of the posterior pituitary gland, using oxidized cellulose as the adsorbent.

METHODS AND RESULTS

In the initial experiment 100 mg. of lyophilized posterior pituitary gland powder was extracted with 100 ml. of 0.25% acetic acid by boiling for 1 minute and filtered. To the extract was added 2 gm. oxidized cellulose (Tennessee-Eastman, 10–12% Carboxyl) previously washed with 1 N HCl, then H_2O and finally 0.1 N HAC. The pH of the mixture was 3.3. The mixture was stirred at room temperature and aliquots removed at various times for determination of oxytocin content by the rooster blood pressure drop method (1939). A similar solution kept at room temperature with stirring for 24 hours but without oxycellulose served as a control and showed no loss in activity due to oxidative changes which may have occurred during the stirring. Results are summarized in Table 1.

TABLE 1

Duration of stirring with oxycellulose	Oxytocin Present (U.S.P. units)	Per Cent Oxytocin Removed from Solution
0	150	0
1 hour	80	53
2 hours	38	75
4 hours	42	72
24 hours	39	75

Attempts to elute the oxytocic factor from the oxycellulose with 0.1 N HCl and 1 N HCl resulted in a recovery of only 30% of the activity.

The optimum amount of oxycellulose necessary to obtain maximum adsorption was determined by stirring 100 ml. posterior pituitary extract containing 100 U.S.P. units of oxytocic activity with varying quantities of oxidized cellulose for a period of 5 hours at pH 3.3. Table 2 presents the data obtained.

TABLE 2

Amount Oxycellulose	Per Cent Adsorption
50 mg	34
200 mg	42
500 mg	55
2000 mg	72

The large amounts of oxycellulose necessary to obtain the maximum adsorption of oxytocin when extracts of posterior pituitary gland were used, were somewhat unexpected in view of the small quantities necessary to secure a similar amount of adsorption of the oxytocic contaminant from ACTH solution. Table 3 indicates this difference. The amount of oxycellulose employed in each case was 200 mg.

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TABLE 3

Fraction	Oxytocic content before adsorption U.S.P. units	Oxytocic content after adsorption U.S.P. units	Per Cent Adsorption
Crude ACTH	4200	1100	74
Crude ACTH	4200	1000	76
Crude ACTH	675	160	76

DISCUSSION

These data indicate that a maximum adsorption of about 76% of the oxytocic factor can be obtained by the use of oxycellulose. However it is to be noted that much smaller amounts of oxycellulose sufficed to remove the oxytocic factor from solutions of ACTH contaminated with oxytocin than from solutions prepared by extraction of the posterior lobe of the pituitary gland.

From the data presented it is not possible to explain the inability to entirely elute the *oxytocic factor from oxycellulose using 0.1 N HCl and 1 N HCl, which observation* contrasts with those of Dumm who found that the antidiuretic factor was completely eluted with 0.1 N HCl. Experiments are now in progress to determine whether buffered salt solutions may accomplish recovery of the oxytocin from the oxycellulose column. The possibility of destruction of the oxytocin during the stirring with oxycellulose by surface denaturation cannot be ignored, although this possibility is remote in view of Dumm's (1952) findings that the antidiuretic factor can be quantitatively adsorbed onto and eluted from oxycellulose columns.

It is also difficult to explain the observation that only about 75% of the oxytocin is adsorbed onto the oxycellulose even when very large amounts of oxycellulose are used.

The results obtained were utilized to accomplish removal of the major portion of oxytocic contamination from the adrenocorticotropic factor isolated from whole hog pituitary glands, and seems to offer a rather effective method for separation of the *bulk of oxytocic contaminant from the adrenocorticotrophic hormone.*

Alginic acid (Kelacid*) was found to have adsorptive properties similar to oxycellulose in its power to adsorb oxytocin and ACTH.

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* Kelco Co., New York City.

HIGH PROGESTATIONAL ACTIVITY OF 19-NORPROGESTERONE

Among the many compounds tested for progestational activity, few have exhibited greater biological potency than progesterone (Ehrenstein, 1948).

Meystre *et al.* (1948) have shown 11-dehydroprogesterone to possess a threefold greater potency than progesterone in the Clauberg test. 17-methylprogesterone A has twice the activity of progesterone using the Corner-Allen assay method (Heuser *et al.*, 1950). Ehrenstein (1944) described the degradative synthesis of 19-norprogesterone from strophanthidin. This material was a resinous substance believed to be a mixture of stereoisomers. In a study of its physiological activity, using the Corner-Allen technique, it was reported to be "fully as active as progesterone, perhaps even more so" (Allen and Ehrenstein, 1944). The synthesis of 19-norprogesterone as the pure crystalline compound by Miramontes *et al.* (1951) made possible the present investigation of its biological activity in the rabbit.[1]

MATERIALS AND METHODS

Rabbit Bioassays:

(a) *Clauberg Test* (Clauberg, 1930). Female New Zealand white rabbits, weighing 600–800 gm., were injected subcutaneously daily with 10 mcgm. alpha estradiol in 0.5 ml. corn oil for 6 days. Following this, the progestagen was injected daily subcutaneously in corn oil solution for 5 days. Twenty-four hours after the last injection the animals were killed. Uteri were weighed and fixed in 4% formalin, sectioned at 8 microns and stained with hematoxylin and eosin.

(b) *Corner-Allen Test* (Corner and Allen, 1929). Sexually mature female New Zealand white rabbits, isolated for 4 weeks were injected subcutaneously with the test compound for 5 days following bilateral ovariectomy and biopsy of the right uterine horn. Approximately 24 hours after the last injection, the animals were killed and the uteri were treated as in (a). The uterine biopsies were taken for histological examination and only those animals were used in which the uterine biopsy showed an estrogen-stimulated endometrium. In each bioassay method, progestational proliferation was rated according to the standard scale of McPhail (1934).

RESULTS AND DISCUSSION

The activity of 19-norprogesterone was compared with that of progesterone in a series of experiments, using the Clauberg method. Table 1 presents data from two of the series.

TABLE 1. PROGESTATIONAL ACTIVITY OF 19-NORPROGESTERONE USING THE CLAUBERG TEST

Series	No. of rabbits	Total dose (mcgm.)*	Progestational proliferation	
			Progesterone	19-norprogesterone
I	3	250	+3, +4, +4	—
	3	500	+4, +4, +3	—
	3	62.5	—	+3, +3, +4
	3	125.0	—	+4, +4, +4
II	3	125	0, 0, 0	—
	3	250	+3, +3, +3	—
	2	31.25	—	+3, +2
	3	62.5	—	+4, +4, +4

* Administered in 1.0 ml. corn oil solution daily over 5 days.

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[1] We are indebted to Chemical Specialties Co. Inc., and Syntex, S.A., for the steroid compounds used in these investigations.

subjected to ether anesthesia during the placing of the needle into the jugular vein but were thereafter kept in activity-restriction cages without anesthesia until death. The dose of cortisone (4 mg. per rat per day) was selected from other experiments (Ingle, Nezamis and Morley, 1951) in which this dose was found to be optimal for sustaining the resistance of the adrenalectomized rat to the severe stress of muscle work.

The data are summarized in Table 1. Cortisone did not significantly affect the resistance of the rat to histamine under these conditions although it is known to suppress the inflammatory response to injury in the normal rat. The amount of histamine required to cause death in the rat under these conditions is remarkable.

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RELATIVE INEFFECTIVENESS OF ELECTRICAL STIMULATION OF THE CERVIX FOR INDUCING PSEUDOPREGNANCIES IN THE MOUSE

The occurrence of pseudopregnancy in the female rat after mating with a vasectomized male was reported by Long and Evans (1922). They also induced this condition by mechanical stimulation of the cervix with glass rods. Parkes (1926) described similar responses in the mouse. Electrical stimulation of the cervix of the rat, by means of small electrodes and a simple induction coil as reported by Shelesnyak (1931) produced a larger percentage of pseudopregnancy than mechanical irritation. This is a report of the relative ineffectiveness of electrical stimulation in producing pseudopregnancy in the mouse.

In a series of experiments requiring pseudopregnant C_3H strain mice, electrical stimulation of the cervix was used. Thirty-six C_3H mice, weighing between 24 and 26 grams, with regular 4–5 day oestrous cycles were selected at the proestrus stage. Intra-vaginal electrical stimulation with Faradic current of approximately 75 volts, peak (Phipps and Bird inductorium, A70-720) was applied blindly to the cervical area for 10 to 15 seconds. No attempt was made to visualize the os cervix for precise placing of the electrodes since in the rat this has not been necessary. Only 8 of the 36 mice became pseudopregnant (averaging 11 days duration) and in 26 of the failures which were stimulated a second time at the next proestrum only 4. Thus out of 62 attempts there were 50 failures. A second group of 40 C_3H mice was treated, using an electronic stimulator, which generated spike waves similar to the inductorium, 105 volts 50 cycles per second. Only 10 of the 40 became pseudopregnant. In the combined series of C_3H mice, 76 females where stimulated at proestrous or early oestrous and after 102 attempts, 22 pseudopregnancies were produced.

To determine whether this low percentage of pseudopregnancies following electrical stimulation of the cervix was a strain or a species difference, the same procedure was carried out on 58 Swiss mice (of which 16 were stimulated on two or more successive cycles). In 22 instances, the stimulus was through the use of the induction coil; in 52, with the electronic stimulator. In the combined group of 58 Swiss mice in which 75

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stimulations were made the number of pseudopregnancies which resulted was 19; and thus the response was similar to that of the C_3H strain.

It appears that a species difference exists between the mouse and the rat in respect to the response of the female to electrical stimulation of the cervical areas during proestrun. In the rat over 90% become pseudopregnant (Shelesnyak, 1931), while in the mouse less than 26% do.

Acknowledgement

Our thanks are due to Miss Myra Arshavsky for technical assistance.

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THE AMERICAN GOITER ASSOCIATION

1953 ANNUAL MEETING

The 1953 Annual Meeting will be held May 7, 8 and 9, 1953, in the Drake Hotel, Chicago, Illinois. The program for the three day meeting will consist of papers and discussions dealing with goiter and other diseases of the thyroid gland.

THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

THE LAURENTIAN HORMONE CONFERENCE

The Laurentian Hormone Conference of the AAAS will hold its 1953 annual meeting at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 6–11. Interested investigators and specialists in the hormone field may apply for attendance by writing to the Committee on Arrangements, 222 Maple Avenue, Shrewsbury, Mass., for application blanks. Since accommodations at the hotel necessarily limit the attendance, only those persons submitting applications can be considered. The application blanks must be received by the Committee no later than *May 15* in order to insure issuance of invitations as soon as possible thereafter.

The following program has been arranged:

I. *Nervous system-hormone interrelationships*
  The central nervous system and stress-induced eosinopenia.
    Dr. R. W. Porter, Univ. of Calif. School of Medicine at Los Angeles
  Studies of brain metabolism and electrical activity in relation to adrenocortical physiology.
    Dr. Hudson Hoagland, The Worcester Foundation for Experimental Biology
  Effects of hormones on cerebral function.
    Dr. D. M. Woodbury, Univ. of Utah School of Medicine

II. *Thyroid Hormone physiology and biochemistry*
  Triiodothyronine in relation to thyroid physiology.
    Drs. J. Gross and R. Pitt-Rivers, National Inst. for Medical Research, London, and State Univ. of New York Medical Center
  Metabolic effects of thyroid hormones *in vitro.*
    Drs. H. A. Lardy and G. Feldott, Univ. of Wisconsin School of Medicine

III. *Comparative endocrinology*
  Endocrine mechanisms in the life of insects.
    Dr. D. Bodenstein, U. S. Army Chemical Center, Maryland
  Hormones produced by neurosecretory cells.
    Drs. Ernst and Berta Scharrer, Univ. of Colorado Medical Center

IV. *Protein Hormones*
  The preparation and chemistry of crystalline insulin.
    Dr. R. G. Romans, Connaught Medical Research Laboratory
  The chemistry of ACTH.
    Drs. E. E. Hays and W. F. White, The Armour Research Laboratories

V. *The role of hormones in blood and blood-forming organs*
  Endocrine factors and radiation-induced lymphoid tumors of mice.
    Dr. Henry S. Kaplan, Stanford Univ. School of Medicine
  Endocrine influences upon the formed elements of blood and blood-forming organs.
    Dr. Albert S. Gordon, Washington Square College, New York Univ.

VI. *Aspects of Clinical Endocrinology*
  Some problems related to ovarian function and to pregnancy.
    Dr. B. Zondek, Rothschild Hadassah Univ. Hospital, Jerusalem
  Clinical studies on electrolyte and fluid metabolism.
    Dr. Rolf Luft, Serafimerlasarettet, Stockholm
  Endocrine-metabolic studies in man.
    Dr. J. W. Conn, Univ. of Michigan School of Medicine

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THE RESPONSE OF THE SUCCINOXIDASE SYSTEM OF RAT LIVER HOMOGENATES TO A SINGLE DOSE OF THYROXINE

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INTRODUCTION

THE *in vivo* effect of thyroxine on the metabolic rate of whole animals, on the oxygen consumption of isolated tissues and on the activity of various enzyme systems has been reviewed by Barker (1951). The mechanism of thyroxine action has been studied most generally in chronically thyroxinized animals. Barker, Kiely and Lipner (1949) have indicated the time lag between the administration of thyroxine and the first measurable increase in the oxygen consumption of the whole animal. Barker and Klitgaard (1952) have demonstrated the unequal response of the tissues of rats to thyroxine. The earliest response elicited occurred 6 hours following a single injection of thyroxine, and liver slices responded in 12 hours. An understanding of the mechanism of thyroxine action involves the elucidation of the cellular processes responding to thyroxine prior to the changes which have been recorded.

This study is concerned with the time response of the liver succinoxidase system of thyroidectomized rats to thyroxine. An analysis of the succinoxidase system of cell granules was performed simultaneously with the study of this enzyme system in rat whole liver homogenates.

METHODS

Male rats of the Sprague-Dawley strain, weighing 300–350 grams, were used in all the experiments included in this study. These animals were maintained on a 5-hour feeding regimen (Barker, 1949) and were usually post-absorptive at sacrifice. The animals were exsanguinated under ether anesthesia by withdrawing 8 to 10 ml. of blood

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[1] Baxter Laboratories Fellow 1951–52.

from the abdominal aorta into an oxalated syringe. The liver was then perfused through the portal vein with 100 ml. of cold Krebs-Ringer solution. A blotted, weighed portion of liver was minced with scissors and homogenized with 2 volumes of 0.25 M sucrose solution in an all glass homogenizer (Potter and Elvehjem, 1936). The homogenate was centrifuged for 5 minutes at 600× gravity and the supernatant decanted into a graduated cylinder. The residue was rehomogenized in 2 volumes of the sucrose solution and then added to the supernatant in the cylinder. The final dilution was such that 1.0 gram of liver was contained in 10 ml. of homogenate suspension.

The fractionation procedure employed was essentially that described by Schneider and Hogeboom (1950).[2] The entire procedure, from the time the liver was excised until the enzyme assay was performed, was carried out at a temperature of 2° to 4° C.

The succinoxidase activity of rat liver homogenates was studied in 5 groups of rats: *normal controls, thyroidectomized controls and 3 groups of thyroidectomized rats* given 6.12 mg./kg. of thyroxine (in 0.02 N NaOH, 0.9 per cent NaCl solution) at 12, 24, and 96 hours previous to sacrifice. The method employed for the assay of succinoxidase is that described by Potter (1949) with the following 2 modifications: (1) it was found unnecessary to perform this assay at 2 enzyme concentrations and (2) determinations of endogenous oxygen consumption were omitted. A series of preliminary experiments indicated no difference in metabolism as calculated from the 2 enzyme concentrations and no endogeneous oxygen consumption. The metabolic activity is expressed as μl. of oxygen consumed per mg. equivalent of liver homogenate per hour.

RESULTS

A summary of the data indicating the effect of thyroxine on the succinoxidase system of rat liver homogenates and cell fractions is presented in Table 1. This system in the thyroidectomized rat liver homogenate is

TABLE 1. THE MEAN SUCCINOXIDASE ACTIVITY OF RAT LIVER HOMOGENATES AND CELL FRACTIONS FOLLOWING A SINGLE INJECTION OF THYROXINE TO THYROIDECTOMIZED RATS

	Normal	Tx	Tx 12 hr. T	Tx 24 hr. T	Tx 96 hr. T
No. of animals	12	13	7	7	10*
Homogenate†	22.5	20.6	24.1	23.0	28.8
S.D.	±1.85	±2.55	±3.62	±1.82	±2.90
Nuclear	4.2	4.5	3.9	4.9	3.2
S.D.	±1.59	±2.10	±2.10	±2.30	±0.42
Mitochondrial	14.3	12.8	15.6	13.1	22.1
S.D.	±2.4	±0.98	±1.2	±0.6	±0.64
Supernatant	2.0	1.8	2.9	2.5	2.4
S.D.	±0.70	±0.84	±0.74	±0.58	±0.56
Sum of Fractions	20.5	18.1	22.4	20.5	27.7

* The livers of 8 animals were used in the fractionation study.

† The means represent μl O_2/mg. wet weight liver or equivalent/hr.

Abbreviations: Tx = thyroidectomized. T = number of hours after a single subcutaneous injection of thyroxine on 6.12 mg./Kg. level.

S.D. = estimated standard deviation of the individual observations from the mean.

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[2] The fractions were prepared in an International Refrigerated Centrifuge, Model PR-I.

approximately 8% less active than in normal controls. Administration of thyroxine resulted in elevation of this level of activity, the 12-hour thyroxinized group showing an increase of approximately 7%, the 24-hour group of approximately 2% and the 96-hour group of approximately 28%.

The activity of this enzyme system in mitochondrial fractions varied with the thyroid status of the animal in a manner similar to that described for the whole homogenate. The nuclear and supernatant fractions possessed succinoxidase activities which, despite alterations in thyroid status of the animals, showed little change.

It should be emphasized that the fractions were not pure preparations since the nuclear fraction was found, by microscopic observation, to be contaminated with small quantities of intact cells, connective tissue, cellular constituents (which were not characterized) and erythrocytes. The mitochondrial or large granule fraction was contaminated to some extent with small granules and occasionally with erythrocytes and nuclei. The final supernatant fraction consisted of the microsomes or small granules, and the soluble constituents of the cell.

Statistical analysis of the data in Table 1 for the whole homogenate succinoxidase activity is presented in Table 2. The data for the fractions were also analyzed statistically. No further information than that obtained from the whole homogenate analysis was discernible. This latter analysis has, therefore, not been included.

TABLE 2. STATISTICAL ANALYSIS OF RAT LIVER HOMOGENATE SUCCINOXIDASE

	No. of animals	Mean μl. O_2/mg. homogenate/hr.	S.D.	t value	Level of significance
					%
I Normal control	12	22.5	1.83		
II Tx	13	20.6	2.55		
III Tx 12-hour T	7	24.1	3.62		
IV Tx 24-hour T	7	23.0	1.83		
V Tx 96-hour T	10	28.8	2.90		
I vs. II				2.15	4.69
I vs. III				1.09	28.66
I vs. IV				0.57	55.64
I vs. V				5.95	0.00
II vs. III				2.27	3.36
II vs. IV				2.42	2.74
II vs. V				7.08	0.00

Abbreviations same as Table 1.

From Table 2 it is apparent that the difference in activity of the succinoxidase system of liver homogenates of normal controls as compared with thyroidectomized rats is significant at the 5% level of confidence. There are no significant differences between the activity of the normal controls and that of the 12-hour or 24-hour thyroxinized animals. The difference in activity between the normal controls and the 96-hour thyroxinized rats is highly significant.

The mitochondrial fractions contributed the following percentages to the whole homogenate succinoxidase activities: normal, 63.4; thyroidectomized, 61.9; 12-hour thyroxinized thyroidectomized, 63.7; 24-hour thyroxinized thyroidectomized 57.2; 96-hour thyroxinized thyroidectomized 75.0. The mitochondrial fraction is primarily responsible for the succinoxidase activity of the cell, since the total recovered activity of the fractions was 91.2, 92.6, 92.4, 89.3 and 94.4 per cent for these groups respectively.

DISCUSSION

The conditions for the assay of the succinoxidase system require that the limiting enzyme in the system: succinic dehydrogenase: cytochrome-c: cytochrome oxidase be the succinic dehydrogenase. Tipton and Nixon (1946) have demonstrated that the cytochrome oxidase system of liver homogenates prepared from normal, thyroidectomized or thyroxinized rats is present in great excess over the succinoxidase system. Schneider, Claude and Hogeboom (1948) have indicated that cytochrome-c is present in excess in the livers of normal rats. However, Drabkin (1950) found that the cytochrome-c content of rat liver diminishes after thyroidectomy and increases in hyperthyroidism. The addition of exogenous cytochrome-c in the assay obviates the possibility of this enzyme being present in deficient quantities. The requirement for exogenous cytochrome-c by the thyroidectomized rat liver succinoxidase system appears to diminish with time after thyroxinization.[3]

Schneider and Hogeboom (1950) have found that the succinoxidase system is primarily contained in the mitochondrial fraction of the rat liver. This localization is unaltered by modification of thyroid status (Table 1). The succinoxidase activity present in the nuclear and supernatant fractions probably indicates the difficulty in achieving preparations uncontaminated by mitochondria.

The response of the thyroidectomized rat liver succinoxidase system to thyroxine may be described as a triphasic response involving return to the control level of activity, plateau at this level, followed by further increase in activity. The first phase of the response may be due to reactivation of enzymes already present but inactive. The second and third phases are probably related. The plateau may represent the time required for the synthesis of additional enzyme. The further increase in activity probably reflects the increasing concentration of enzymes.

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[3] Unpublished data.

SUMMARY

The activity of the succinoxidase system of rat liver homogenates has been studied in normal control and thyroidectomized rats and compared with that of thyroidectomized rats 12, 24 and 96 hours following the administration of thyroxine.

Under all the conditions studied the succinoxidase system was predominantly present in the mitochondrial fraction. The latter was prepared by differential centrifugation of rat liver homogenates suspended in isotonic sucrose.

The liver homogenate succinoxidase activity of thyroidectomized rats returns to control levels within 12 hours following thyroxine administration and remains at this level for an additional 12 hours before any further increase in activity occurs. The implications of this finding are discussed.

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THE INFLUENCE OF THE ADRENAL CORTEX ON AEROBIC GLYCOLYSIS BY NORMAL AND MALIGNANT RAT LYMPHOID TISSUE[1]

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THE regulatory influence of the adrenal cortical secretions on the structure and function of normal lymphoid tissue has been demonstrated by White and Dougherty (cf., White, 1947–48). In addition, a number of studies have indicated that the growth of lymphoid tumors may also be under the control of the adrenal cortex (Heilman and Kendall, 1944; Murphy and Sturm, 1944; Sturm and Murphy, 1944; Law and Speirs, 1947; Diller, Beck, and Blauch, 1948; Pearson, Eliel, and Rawson, 1950; Ingle and Nezamis, 1951; Emerson, 1951).

These observations suggested that the metabolic behavior of normal and malignant lymphoid tissue might be influenced by variations in pituitary-adrenal cortical activity. Accordingly, investigations were undertaken of the influence of the adrenal cortex on the respiratory and aerobic glycolytic activity of normal and malignant rat lymphoid tissue, *in vitro*. These metabolic processes in normal lymphoid tissue were unaffected by the adrenal cortical secretions. However, surviving lymphosarcoma tissue from rats pre-treated with adrenal cortical hormones or ACTH exhibited an increased rate of glucose utilization. This was not accompanied by a change in lactate accumulation or in total oxidative metabolism in the tumor tissue as a result of hormone treatment.

METHODS

The animals supplying normal and malignant lymphoid tissue for the *in vitro* investigations were adult male Sprague-Dawley rats, weighing 250–300 gm. All animals were maintained on Purina laboratory chow and drinking water *ad libitum*, with a weekly supplement of fresh lettuce.

Bilateral adrenalectomy, where performed, was carried out via the dorsal approach 4 to 8 days before autopsy. The animals were then given 1 per cent NaCl to drink instead of tap water. Hormone-treated rats were injected intraperitoneally with ACTH

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(Actarmour) or ACE (Wilson's aqueous adrenal cortical extract) 2 hours before tissue sampling. The dose of ACTH employed was equivalent to 4 mg. of the Armour standard, LA-1-A, per 100 gm. body weight. Usually, 2 ml. of ACE was the dose used per rat.

The tissues studied included normal spleen, thymus, and mesenteric lymph nodes,[3] and a transplantable lymphosarcoma maintained by subcutaneous passage in Sprague-Dawley rats. This tumor had originally been produced and carried in Wistar animals.[4]

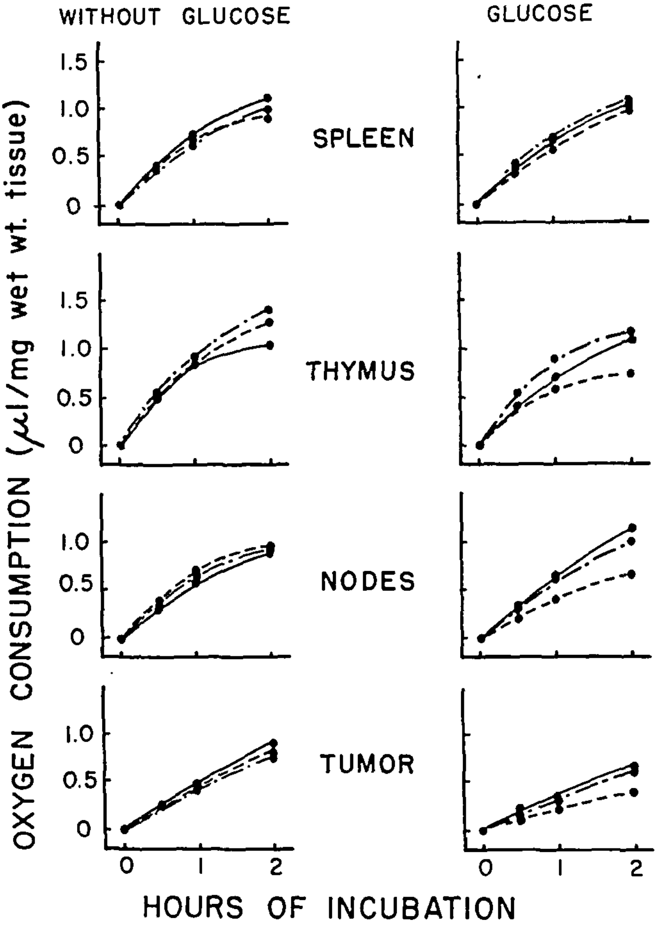

FIG. 1. The influence of the adrenal cortex on the respiration of normal rat lymphoid tissue and a transplantable lymphosarcoma. Solid lines represent tissue obtained from control animals; dotted lines, adrenalectomized; dashed lines, hormone-injected. Each point is the average of 8 to 15 determinations.

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[3] "Mesenteric lymph nodes" refers to the mesenteric chain of nodes dissected free of fat and connective tissue prior to mincing.

[4] We are indebted to Dr. Lloyd Law of the National Institutes of Health for supplying us with this tumor. Histological confirmation of the lymphomatous character of the tumor in Sprague-Dawley recipients was obtained through the generous assistance of Dr. B. G. Lamson, Department of Pathology, School of Medicine, University of California at Los Angeles.

Surviving tissues were obtained from animals which had been exsanguinated from the abdominal aorta while under light nembutal anesthesia. These tissues were rapidly removed, chilled, and minced free-hand with a single-edge razor blade. Approximately 100 mg. of tissue were incubated in 2 ml. of incubation medium at 38° C. Respiration studies were carried out in Krebs-Ringer-phosphate buffer in Warburg flasks which had been gassed with 100 per cent oxygen. In some instances glucose was added to the medium (0.15%), and glucose utilization measurements were made. A number of metabolic studies were conducted in rat serum with a constant stream of 95% oxygen, 5% carbon dioxide passing through the flask. The serum was obtained about one hour before incubation from rats similar to those supplying normal tissue. The glucose content of this serum was approximately 130 to 160 mg. per 100 ml.

After varying periods of incubation, the flask contents were centrifuged at high speed. Aliquots of the supernatant were analyzed for carbohydrate by the anthrone procedure after protein precipitation with trichloroacetic acid (Morris, 1948). Lactate was determined by the method of Barker and Summerson (1941) and amino acid nitrogen by the method of Frame, Russell, and Wilhelmi (1943) on tungstic acid filtrates of the medium.

RESULTS

1. *Respiration of normal and malignant lymphoid tissue*

Comparison was made of the respiration rates of normal and malignant lymphoid tissue minces obtained from adrenalectomized, hormone-injected, and control rats. Samples of spleen, thymus, and mesenteric lymph nodes were obtained from non-tumorous animals of the same age, weight, and dietary history as the rats supplying the lymphosarcoma tissue. The results obtained (Fig. 1) are expressed in terms of µl. oxygen consumed per mg. wet weight of tissue during the two-hour incubation period. In several instances, tissue water determinations were made. The Q_{O_2} values calculated on the usual dry tissue weight basis were substantially in agreement with the oxygen consumption values depicted.

It will be noted that both normal and malignant lymphoid tissues exhibited similar rates of respiration in the absence of glucose in the medium. The addition of glucose to the medium (0.15%) appeared to have no significant effect on the oxygen consumption of normal lymphoid tissue, but depressed the respiration of tumor minces. Hormone treatment (ACE or ACTH) uniformly had no effect on the oxygen consumption of normal or malignant tissues. Adrenalectomy tended to depress respiration only when glucose was present in the medium.

2. *Glucose disappearance and lactate accumulation*

The aerobic disappearance of glucose in the presence of surviving normal and malignant lymphoid tissue was studied in phosphate buffer fortified with glucose (0.15%) under an atmosphere of 100% oxygen and also in a rat serum medium under an atmosphere of 95% oxygen, 5% carbon dioxide. The effect on glucose utilization of pretreatment of the rats with

ACE or ACTH, or of previous adrenalectomy, was also followed.

The results obtained in serum and buffer were similar. Figure 2 and Table 1 show the effect of the adrenal cortical secretions on aerobic glucose

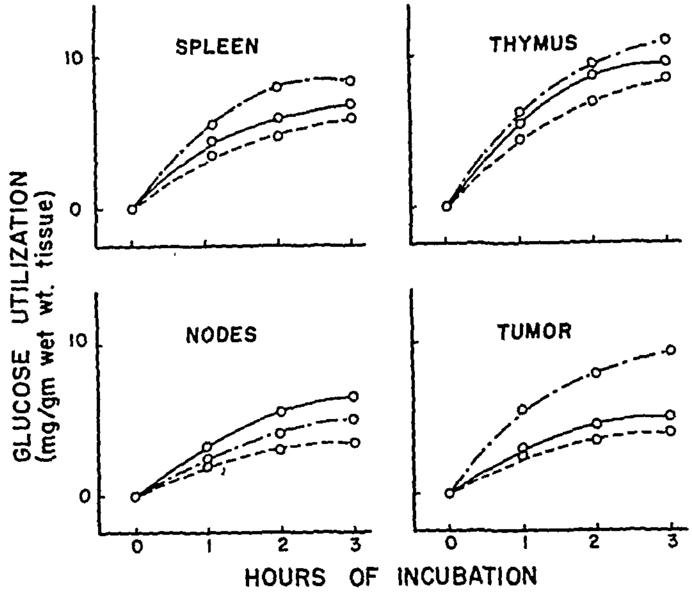

Fig. 2. The influence of the adrenal cortex on the aerobic utilization of glucose by normal rat lymphoid tissue and a transplantable lymphosarcoma. Incubations were conducted in Krebs-Ringer-phosphate buffer medium. Solid lines represent control animals; dotted lines, adrenalectomized; dashed lines, hormone-injected. Each point is the average of 6 to 12 determinations.

Table 1. The influence of the adrenal cortex on aerobic glycolysis by normal rat lymphoid tissue and a transplantable lymphosarcoma*

Tissue	Treatment	Number of rats	Glucose utilization rate†	Lactate accumulation† (as lactic acid)
Mesenteric Lymph Nodes	Control	13	1.76 ±0.12	0.80 ±0.11
	ACTH or ACE	9	1.78 ±0.13	0.72 ±0.08
	Adrx	10	1.59 ±0.09	0.56 ±0.10
Lymphosarcoma	Control	9	2.03 ±0.15	1.50 ±0.20
	ACTH or ACE	10	4.22 ±0.20	1.63 ±0.15
	Adrx	7	1.97 ±0.12	1.39 ±0.13

* All incubations were carried out for 2 hours in a rat serum medium at 38° C. with a constant stream of 95 per cent oxygen, 5 per cent carbon dioxide passing through the flask.

† The figures shown are expressed as mg. per hour per gm. wet wt. tissue (average ± standard error).

utilization by normal lymphoid tissues and by lymphosarcoma tissue in phosphate buffer and serum, respectively. Adrenalectomy, ACE and ACTH were without effect on glucose utilization by normal lymphoid tissue. On the other hand, adrenal cortical hormones stimulated the glucose utilization rate of the tumor tissue under these conditions. The accumulation of lactic acid during the incubation of normal mesenteric lymph nodes and lymphosarcoma tissue in serum is indicated in Table 1. Lactate accumulation was considerably higher in the tumor tissue incubates. The adrenal cortical secretions produced no striking effect on lactate accumulation in either normal or malignant lymphoid tissue incubates.

DISCUSSION

The profound histological changes which occur in normal lymphoid structures as a result of pituitary-adrenal cortical hyperactivity (Dougherty and White 1945) were not accompanied in the present investigations by striking alterations in the determined metabolic activities of normal rat lymphoid tissues *in vitro*. Previous adrenalectomy, it is true, tended to depress lymphoid tissue metabolism. This depression, however, cannot certainly be ascribed to a lack of circulating adrenal cortical hormones in view of the absence of an accentuating effect of hormone-treatment.

Perhaps of more interest were the observations made of the influence of the adrenal cortical secretions on the aerobic metabolism of malignant lymphoid tissue. The addition of glucose to tumor incubates significantly depressed the respiration of this tissue, but had little or no effect on the respiration of normal lymphoid tissue metabolism. The obvious inference was that glucose supplementation of the medium permitted the tumor to obtain more of its energy from glycolysis. This was borne out by the observation that lymphosarcoma tissue converted the major proportion of added glucose to lactate even under aerobic conditions.

The dependence of tumors of all kinds on glycolytic mechanisms for energy has long been recognized (cf. Warburg, 1930). In the present studies, this dependence may well have been related to the stimulating effect of the adrenal cortical secretions on the aerobic utilization of glucose by the tumor. Thus, the previous administration of ACTH or ACE was observed to accelerate the disappearance of glucose in the presence of surviving lymphosarcoma tissue. However, a concomitant increase in lactate accumulation or oxygen consumption did *not* occur. It seems likely, therefore, that the accentuated utilization of carbohydrate resulting from hormone administration was accompanied by the sparing of endogenous substrate.

The relationship of the above observations to the growth-arresting effect of cortisone and ACTH on lymphoid tumors is at present obscure. It is possible that an increased requirement for carbohydrate under these conditions may be the limiting factor responsible for tumor regression.

SUMMARY

Adrenalectomy, ACTH, and adrenal cortical extract (ACE) were without striking effect on the respiration or aerobic glycolytic activity of surviving normal rat lymphoid tissue, including spleen, thymus, and mesenteric lymph nodes.

In contrast, the aerobic utilization of glucose by lymphosarcoma tissue *in vitro* was found to be significantly elevated by previous treatment of the animal supplying the tissue with ACTH or ACE. This increase was not accompanied by a change in lactate accumulation or total oxidative metabolism. The possible relationship of these findings to the growth-arresting effect of the adrenal cortical secretions on lymphoid tumors is discussed.

Acknowledgment

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EFFECTS OF AMPHENONE "B" ON THYROID AND ADRENAL FUNCTIONS[1]

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AMPHENONE "B"[3] (Allen and Corwin, 1951), a substituted desoxybenzoin, given orally or subcutaneously, produces marked enlargement of rat thyroid and adrenal glands (Hertz, Allen and Tullner, 1950; Hertz, Tullner and Allen, 1951; Hogness, Williams and Lance, 1952). The thyroid enlargement is associated with reduction in I^{131} concentration (Hogness, Williams and Lance, 1952) and a lowered metabolic rate (Holtkamp, Heming and Sax, 1952). The thyromegalic effects are not found in hypophysectomized rats (Hertz, Allen and Tullner, 1950) or in those given supplementary thyroid extract (Hertz, Tullner and Allen, 1951). The adrenomegaly produced by Amphenone "B" is associated with increase in cholesterol concentration (Hogness *et al.*, 1952) without evidence of decreased adrenal function (Hertz *et al.*, 1951; Hogness *et al.*, 1952; Heming *et al.*, 1952). As in the case of the thyroid, adrenal enlargement is not found in hypophysectomized animals (Hertz *et al.*, 1950) or in those given cortisone (Hertz *et al.*, 1951).

In the course of previous studies with this compound certain questions arose. A marked reduction of radioiodine concentration in the thyroid occurred following the injection of Amphenone "B." Although this probably represented inhibition of I^{131} uptake, the possibility of an increased rate of release of iodine from the gland existed. In the case of the adrenal, most evidence indicated normal function following Amphenone "B" administration. However, the possibility of a partial block of steroid output remained (Hertz, Tullner and Allen, 1951). Because of possible clinical uses of a compound with such marked effects upon both thyroid and adrenal function, it seemed important to investigate these problems, and it

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[3] Kindly supplied by Dr. Arthur E. Heming, Smith Kline and French Laboratories, Philadelphia, Pennsylvania.

was with this purpose in mind that the following experiments were designed.

METHODS

Sprague-Dawley rats were used in all experiments; age, weight and sex varied with the experiment but were the same within any given group and its controls. The animals were fed Purina laboratory chow and were kept at constant temperature of 78° F. Amphenone "B" was administered by subcutaneous injection, unless otherwise noted.

Thyroid: In order to determine more definitely whether Amphenone "B" exerted an inhibitory or secretory effect, the following experiments were conducted. First, thyroid glands were removed from a series of male rats weighing approximately 200 grams and placed in Krebs-Ringer solution, pH 7.4, containing Amphenone "B" in molar concentrations ranging from 10^{-3} through 10^{-7}. The thyroids from four animals were used at each concentration. Immediately upon introduction of the glands into the solutions, 10 μc. of I^{131} was added to each flask. The flasks were incubated in a Dubnoff shaker at 37° for one hour, whereupon the glands were removed, washed, placed in 6N NaOH, homogenized, and radioactivity assayed by means of a Geiger-Mueller counting system. Results were expressed as the per cent of I^{131} uptake exhibited by the control glands. For purposes of comparison an identical experiment was carried out, substituting propylthiouracil for Amphenone "B."

In a second experiment four 100 gm. male rats were given 20 mg. of Amphenone "B" in 0.5 ml. of normal saline, intravenously. After one hour, these rats and four controls were given 25 μc. of I^{131} in saline subcutaneously. Four hours later the rats were sacrificed and radioactivity in each lobe of the thyroid determined. Results were expressed as per cent of injected I^{131} per mg. of thyroid tissue.

The following experiment was designed to determine whether Amphenone "B" caused evacuation of radioiodine from the thyroid. Fifteen male rats weighing 200 to 250 gm. were given 25 μc. of I^{131} subcutaneously. Five hours later the animals were sacrificed, the thyroid glands removed and the two lobes separated. One lobe from each animal was incubated in Krebs-Ringer solution containing 10^{-3} M Amphenone "B," while the other lobe was incubated in Krebs-Ringer solution containing 10^{-3} M propylthiouracil; all flasks contained 0.001% KI in order to block I^{131} reincorporation. After incubation for 90 minutes in a Dubnoff shaker, the glands were placed in 6N NaOH, homogenized and radioactivity assayed.

Another experiment similar to the above was conducted; however, in this case one half of the lobes were incubated in Krebs-Ringer solution containing KI and propylthiouracil and the other half were incubated in Krebs-Ringer solution containing KI alone, as a control.

Adrenal: To establish more conclusively (a) whether Amphenone "B"-induced adrenomegaly is associated with adrenal blockade or a normal or increased output of adrenal steroids and (b) whether Amphenone "B" exerts an immediate effect on the adrenal, similar to ACTH, the following two experiments were devised.

Eighteen 90 gm. female rats were given Amphenone "B" twice daily for four and one-half days. The dosage was 10 mg./100 gm. body weight and the drug was administered subcutaneously in 0.5 ml. of physiological saline, pH 4.0. Twelve hours before sacrifice all animals were given the last injection of Amphenone "B" and food was withdrawn. Four hours before death six of the animals were given a subcutaneous injection of 1 I.U. of ACTH,[4] six were placed in the cold (for 4 hours) and six served as controls. At the was 26 mg./100 gm. body weight. This difference is significant ($P < 0.01$). Neither ACTH nor exposure to cold altered the adrenal weight.

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[4] Kindly supplied by Dr. Grosvenor Bissell, Armour and Co., Chicago, Illinois.

The center graph in Figure 4 represents the adrenal ascorbic acid content. Whereas there appears to be some increase in ascorbic acid concentration in the Amphenone "B" treated animals as compared with the controls (mean values 0.446 and 0.400 mg./100 mg. of adrenal respectively) this difference is not statistically significant. However, within each group the differences in adrenal ascorbic acid concentration following ACTH or cold stimulation were highly significant. Injection of 1 I.U. of ACTH into Amphenone "B" treated rats resulted in a fall in adrenal ascorbic acid

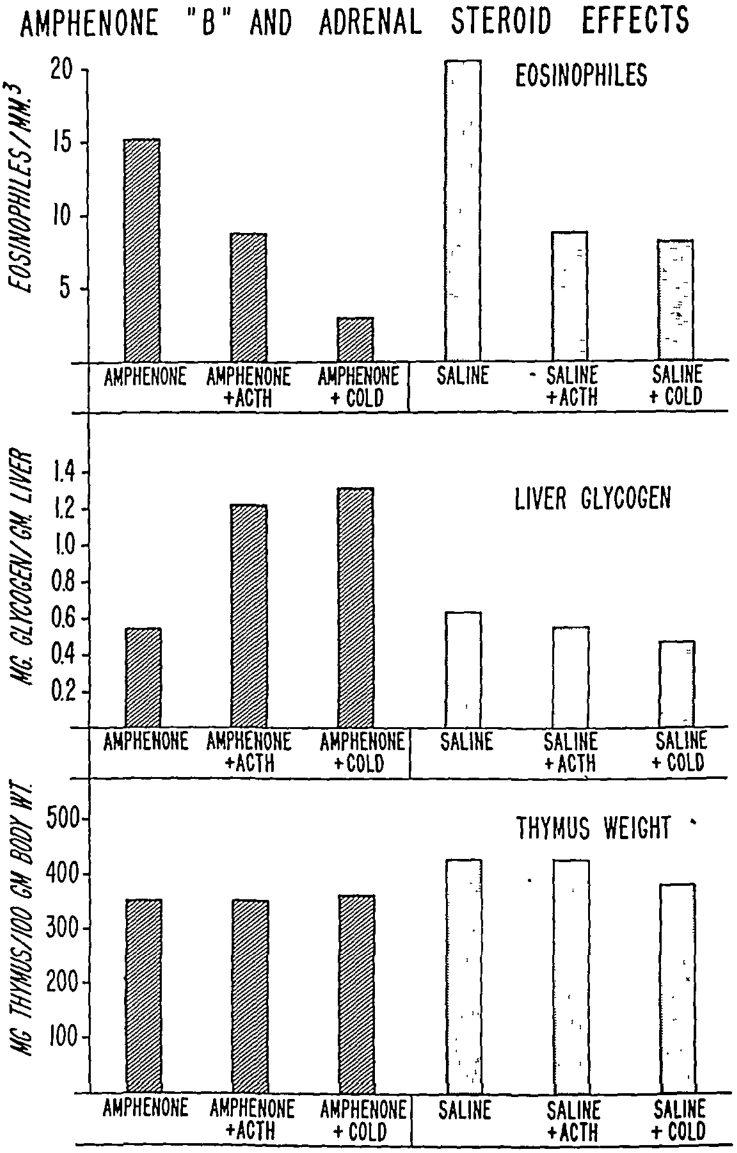

FIG. 5

from a mean of 0.446 mg./100 mg. of adrenal to 0.356 mg. ($P < 0.001$) and subjection to cold for four hours produced a more marked decrease to a mean of 0.277 mg./100 mg. of adrenal. In the saline controls, under conditions of both ACTH and cold stimulation, the ascorbic acid concentration fell from a mean of 0.400 to 0.344 and 0.345 respectively ($P < 0.03$). These observations are of interest in that in Amphenone "B" treated animals, ACTH or cold stimulation produced a greater adrenal response than was seen in the saline controls. It would also appear that four hours in the cold was a greater stimulus than subcutaneous administration of 1 I.U. of ACTH.

The adrenal cholesterol concentration seen in the lower graph in Figure 4 paralleled the observations on ascorbic acid. A more than two fold increase in cholesterol concentration was observed in the Amphenone "B" treated animals, confirming previous observations. However, despite this increase, cold stimulus for example, produced a marked fall in cholesterol concentration from a mean of 6.86 mg./100 mg. adrenal to a mean of 5.02 mg. ($P < 0.01$). As may be seen from the graph, this response was of greater magnitude than that seen in the controls. As was the case with the ascorbic acid determinations, cold appeared to produce a greater stimulus than the injection of one I.U. of ACTH.

Figure 4 represents direct measurements of adrenal properties. However, if a stimulus to the adrenal is to be effective it must produce not only changes in the adrenal itself but also an increase in production and secretion of adrenal steroids. Such an increased output might be reflected by changes in (a) circulating eosinophiles (b) liver glycogen content and (c) thymic weight. Figure 5 shows that such changes do occur under the conditions of this experiment.

Following ACTH or cold stimulation there was a significant fall in circulating eosinophiles in both the Amphenone "B" treated animals and the controls. Again, this fall is more pronounced with cold exposure, especially in the case of the Amphenone "B" animals. Although the absolute eosinophile determinations seem somewhat higher in the controls than in the Amphenone "B" animals (20.4 $mm.^3$ as compared with 15.2 $mm.^3$) this difference is not of significance statistically.

Although the fasting liver glycogen is the same in Amphenone "B" treated animals and saline controls, a highly significant increase in liver glycogen occurred when the Amphenone "B" animals were injected with 1 unit of ACTH or subjected to four hours in the cold. A rise of from 0.54 mg. of glycogen per gram of liver to 1.22 and 1.31 mg. respectively, resulted ($P < 0.02$). No such rise occurred in the control group, indicating again that the Amphenone "B" stimulated gland is capable of an even more marked response to ACTH and cold than the controls.

The bottom graph in Figure 5 represents thymic weights in Amphenone "B" and control groups. The mean thymus weight in the control rats was 425 mg./100 gm. body weight, whereas the mean in the Amphenone "B" treated rats was 351 mg. This difference is significant ($P < 0.01$). No significant differences were found within Amphenone "B" and control groups, however.

The results of an acute experiment, in which one injection of Ampehenone "B" was given four hours before death, are shown in Table 2. No differences of any significance were observed between the values obtained in the animals given no injections, those injected with saline, and those to whom Amphenone "B" was administered. This indidates that, with respect to the adrenal, one injection of Amphenone "B" does not exert a measurable immediate effect.

TABLE 2. EFFECT OF SINGLE SUBCUTANEOUS INJECTION OF AMPHENONE "B" ON ADRENALS

	Control	Saline	Amphenone "B"
Adrenal weight (mg./100 gm. body wt.)	30.2	29.4	30.5
Ascorbic acid (mg./100 mg. adrenal)	0.37	0.42	0.31
Cholesterol (mg./100 mg. adrenal)	2.15	2.04	1.87
Thymus weight (mg./100 gm. body wt.)	161	154	169
Eosinophiles (per $mm.^3$)	38.3	34.0	39.2
Liver glycogen (mg./gm. liver)	3.3	3.1	2.3

DISCUSSION

It has been shown that prolonged administration of Amphenone "B" results in thyroid enlargement. The studies reported in this paper show than Amphenone "B," in low concentration, inhibits the incorporation of radioactive iodine into the thyroid both *in vitro* and *in vivo*. On a molar basis Amphenone "B" was shown to be as effective a thyroid blocking agent as is propylthiouracil. Whereas both agents produced marked inhibition of thyroid I^{131} uptake, neither produced any change in the rate of release of iodine.

It is concluded that the increase in thyroid size observed following Amphenone "B" administration occurs on the same basis as with propylthiouracil. It is presumably the result of an increase in circulating TSH brought about by a fall in the blood thyroxine level, resulting from antagonism to its synthesis. This conclusion is supported by the observation of Holtkamp, Heming and Sax (1952) of a lowered oxygen consumption following Amphenone "B" administration.

The observations on the thyroid suggested that the adrenal enlargement and associated rise in cholesterol concentration noted after prolonged Amphenone "B" administration might also be the result of a "blocking" action. Contrary to original expectations however, no diminution in adrenal function was observed. Evidence presented indicates that the adrenals of the Amphenone "B" treated animal are capable of responding to stimulation by secreting an even greater quantity of steroids than those of the controls. In animals receiving Amphenone "B" over a four and one-half day period the administration of 1 I.U. of ACTH or exposure to cold for four hours produced a marked fall in adrenal ascorbic acid and cholesterol concentrations. This was associated with a pronounced rise in liver glycogen and a significant fall in circulating eosinophiles. These changes were more marked in the Amphenone "B" treated animals than in the control group. Therefore, not only are the adrenals of the Amphenone "B" treated animals capable of responding to stimulation but it would seem reasonable to conclude that they respond by secreting a greater quantity of steroids than the controls. Of interest was the observation that, in the Amphenone "B" treated animal, exposure to cold for four hours apparently evokes a greater adrenal response than one I.U. of ACTH administered subcutaneously.

The conclusions reached above appear to be quite the opposite of the situation observed in the case of the thyroid. Thiouracil has been shown to produce an effect on the adrenal (Zarrow and Money, 1949) as well as on the thyroid, but in this instance the changes observed consisted of a true cortical atrophy. Hence, although Amphenone "B" acts like thiouracil where the thyroid is concerned, the action on the adrenal is very different from that of thiouracil.

In a previous experiment (Hogness, Williams and Lance, 1952) it was shown that administration of 35 mg. of Amphenone "B" in four divided doses, over a 24-hour period, produced no change in adrenal size, adrenal cholesterol concentration or thymus size. Therefore Amphenone "B" does not exert an effect like short term administration of ACTH, formaldehyde, or other compounds that produce an acute "alarm" response. The reaction is also unlike that observed after the administration of such substances as erucic acid where an increase in adrenal size and cholesterol content has been noted but no change in steroid secretion was apparent (Carroll, 1951; Carroll and Noble, 1952).

Since Amphenone "B" does not exert a blocking effect on the adrenal and since the increase in adrenal size is not produced in hypophysectomized animals, our results can be explained by postulating that the compound stimulates the anterior pituitary or hypothalamus directly and that the adrenomegaly is the result of an increase in trophic hormone brought about by this stimulus.

This interesting compound has three distinct sites of action. It produces progestational effects (Allen, Hertz and Tullner, 1950) presumably by a direct action on the end organs, since these changes occur even in hypophysectomized animals (Hertz *et al.*, 1950). It also acts directly on the thyroid by inhibiting iodine uptake and hence suppressing thyroid function, and finally, the compound apparently has a direct stimulatory action on the pituitary, producing adrenomegaly in this manner.

SUMMARY

1) Amphenone "B" was shown to inhibit the incorporation of I^{131} into thyroid protein both *in vivo* and *in vitro*. This effect was present when molar concentrations of the inhibitor were as low as 10^{-6}, and the compound was shown to be equally as potent an anti-thyroid substance *in vitro* as propylthiouracil. Neither Amphenone "B" nor propylthiouracil was shown to have any effect on the rate of release of radioiodine from the rat thyroid.

2) Prolonged administration of Amphenone "B" to rats produced adrenomegaly which was associated with increases in adrenal ascorbic acid and cholesterol concentrations. Determinations of liver glycogen, circulating eosinophiles and thymus weight indicated that the enlarged adrenals were capable of responding to ACTH or cold stimulus by secreting an increased quantity of steroids. The response in Amphenone "B" treated animals was even greater than that of the controls.

3) It is concluded that Amphenone "B" has a direct antithyroid "blocking" action but that the effect on the adrenal is stimulatory in nature.

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THE PROTEIN ANABOLIC EFFECT OF TESTOSTERONE PROPIONATE AND ITS RELATIONSHIP TO INSULIN

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SINCE Kochakian and Murlin (1935, 1936, 1937) performed their fundamental experiments on castrate dogs it has been well established that administration of androgenic substances causes a fall in the non-protein nitrogen level (NPN) in blood and at the same time lowers the nitrogen excretion in the urine. It is well known also that insulin has a very similar effect when injected into the diabetic organism (Falkenhausen, 1925, Chaikoff and Forker, 1950)—in other words, insulin and the male sex hormone may, under certain circumstances, exert a similar protein anabolic effect. The interrelationship between testosterone propionate and insulin has therefore been investigated and as a first step the behavior of the NPN and blood sugar levels of normal and depancreatized dogs receiving testosterone has been studied.

EXPERIMENT

This study was carried out on eight well trained female dogs of a weight between 20–25 Kg. Three of them were surgically depancreatized and a standard diet was fed twice a day. Regular (Toronto) insulin was used and administered subcutaneously twice a day in order to keep the urine of the animals sugar-free. Studies were made on 1) normal dogs, 2) totally depancreatized dogs after withdrawal of insulin and on 3) depancreatized dogs receiving various doses of insulin. Each experiment was carried out twice on the same dog, once without testosterone and again after injection of testosterone.

The injections of the male sex hormone (we are grateful to the Ciba Company for supplies of Perandren) were given in the following way: the first morning 50 mg. of testosterone propionate dissolved in Sesame oil were given intramuscularly, the second day 50 mg. in the morning and 25 mg. in the afternoon. The third day the actual experiment was carried out when 4–7 blood samples were drawn from the animal which was fasting for 24 hours before start of the experiment. A dog treated with testosterone was used again only after an interval of at least 4–5 weeks when it was assumed that all injected testosterone had disappeared from the body.

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Blood sugar was determined according to Miller and Van Slyke (1936) and for NPN determinations on whole blood Folin and Wu's (Boström, 1944) colorimetric method was applied. The precipitation of blood proteins was carried out according to a special modification described by Folin (1930). In this not very common form of protein precipitation blood cells are minimally damaged and haemolysis is prevented. Using this modified precipitation method the results are around 12 mg.% lower than figures obtained by the original method of Folin and Wu and even small variations in the NPN level can be detected.

RESULTS

The dose of 125 mg of testosterone propionate injected into a normal dog under the standardized conditions of our experiments proved to be highly effective as demonstrated in Figure 1. A similar depression of the NPN level in blood was obtained also in the other four intact animals. In contrast to normal dogs, the same dose of testosterone injected into our totally depancreatized dogs did not exert any effect on the NPN level when the exogenous supply of insulin was withheld for 24 hours before the start of experiment. Figures 2a and 2b are good illustrations of the negative response of diabetic dogs when only traces of insulin were present in the body. When under the same conditions an arbitrarily chosen dose of insulin was injected into such a diabetic dog a remarkable lowering of the

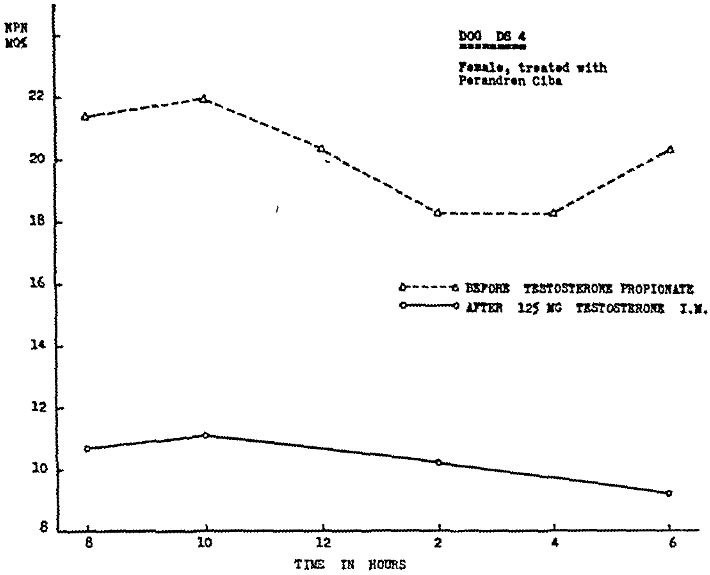

FIG. 1. The normal female dog responds to testosterone by a remarkable lowering of the non-protein nitrogen level in blood.

NPN occurred, as shown in Figure 3, and no further depression of the NPN level could be obtained when the same dose of insulin and testosterone were acting together at the same time. The same observation was made after injections of even smaller amounts of insulin, such as 7, 4 or 2

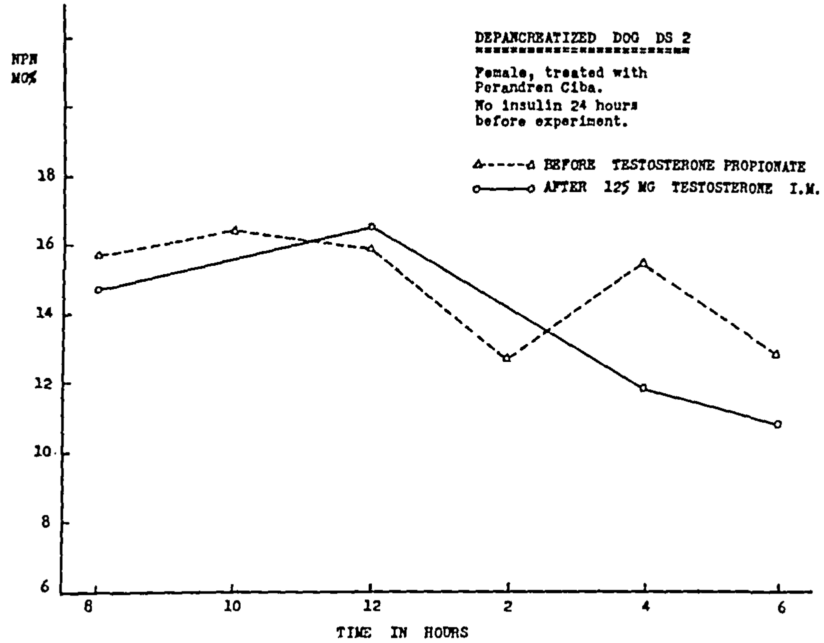

FIG. 2a. The depancreatized dog kept for 24 hours without exogenous insulin exhibits no change of the non-protein nitrogen level after testosterone treatment. Dog DS2.

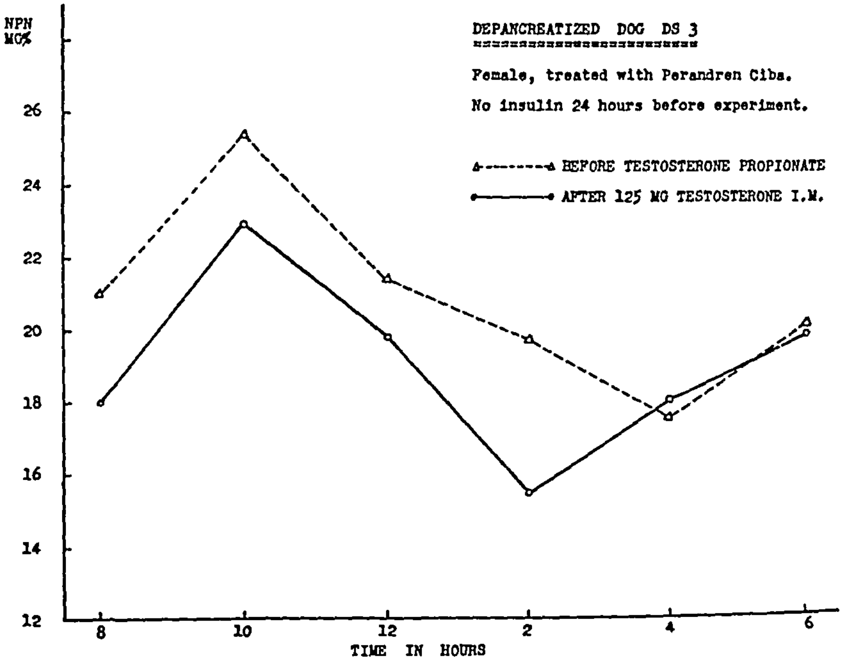

FIG. 2b. Same as 2a. Dog DS3.

units. The fall of the NPN, however, as could be expected *a priori*, after smaller doses of insulin was not so dramatic as shown in Figure 3, but testosterone never influenced the NPN level in either direction in these depancreatized dogs.

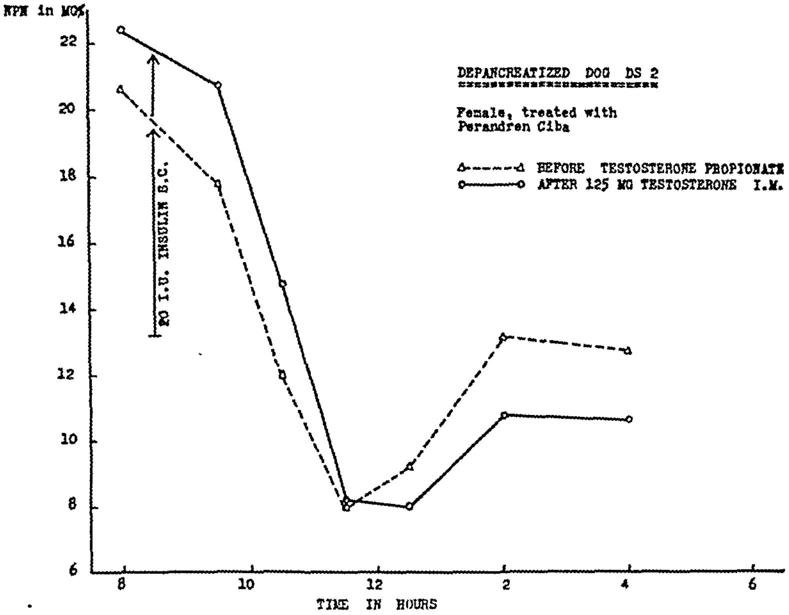

FIG. 3. The diabetic dog responds to insulin by a prompt lowering of the non-protein nitrogen level. Testosterone neither supplements nor counteracts the NPN-lowering effect of insulin.

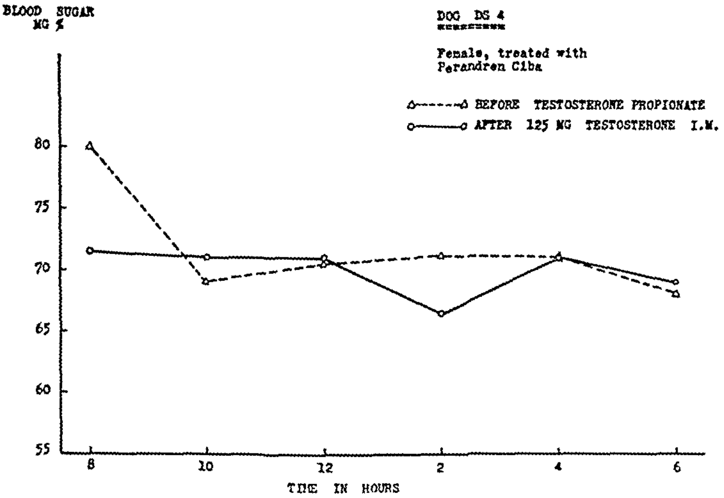

FIG. 4. A standard dose of 125 mg. of testosterone propionate does not affect the blood sugar level of the normal female dog.

CONJOINT EFFECTS OF PYRIDOXINE DEFICIENCY AND ANTERIOR PITUITARY GROWTH HORMONE ADMINISTRATION IN THE RAT

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IT HAS been shown in enzymatic studies (Cammarata and Cohen, 1950; Armstrong, Feldott and Lardy, 1950) and investigations with intact rats (Sheppard and McHenry, 1946; Hawkins, MacFarland and McHenry 1946; Beaton, Ballantyne, Lau, Steckley and McHenry, 1950) that vitamin B_6 affects nitrogen metabolism. Anterior pituitary growth hormone has been stated to promote nitrogen retention (Li and Evans, 1947; Russell, 1951). In order to determine whether the hormone would exert its anabolic influence in rats rendered deficient by dietary restriction of vitamin B_6 and by the administration of the vitamin antagonist, desoxypyridoxine, three studies differing somewhat in experimental detail were performed.

EXPERIMENTAL

In all experiments, albino rats of the Wistar strain were housed in individual screen-bottomed cages and fed either a fat-free, 15% casein diet or a 20% corn oil, 20% casein diet as noted. Except for sucrose, the other ingredients in per cent by weight were common to both regimens: salts mixture (Steenbock and Nelson, 1923) 4, vitaminized casein 4 (the protein content included in the total casein), agar 2, choline 0.2, inositol 0.2 and cod liver oil concentrate .015 (supplying approximately 45 I.U. vitamin A and 11 I.U. vitamin D per rat per day). Sucrose made up the remainder of the diet in each case. The vitaminized casein was a mixture of: 100 mg. thiamine chloride, 100 mg. riboflavin, 400 mg. calcium pantothenate, 400 mg. nicotinic acid, 400 mg. p-aminobenzoic acid, 20 mg. biotin, 20 mg. folic acid and 800 gm. casein. All animals were given daily supplements of 100 μg. desoxypyridoxine per rat per day in the diet; control animals were also provided with 50 μg. pyridoxine hydrochloride per rat per day in the diet. The rats receiving anterior pituitary growth hormone[1] were injected with 1 mg. in 1 cc. saline per rat per day in a single dose. Injections of saline of the same volume were given to the remaining rats.

Alanine-load tests were carried out with both vitamin B_6-deprived and

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[1] Armour Laboratories 22KR1.

pyridoxine-supplemented groups by injecting, intraperitoneally, 15.2% DL-alanine in water in quantity to provide 0.5 mg. nitrogen per gm. body weight based on the average body weight per group. One group of animals on each treatment was sacrificed at set intervals of time following the amino-acid administration. Zero hour groups received 5 cc. 0.9% saline per rat by the intraperitoneal route and were sacrificed immediately. Subsequent to anesthetization with nembutal, blood was removed from the exposed heart, heparinized, and pooled for each group. The following analyses were done on the pooled samples: urea (Archibald, 1945), amino nitrogen (Frame, Russel and Wilhelmi, 1943), plasma free glutamic acid and glutamine (Prescott and Waelsch, 1947), sugar (Nelson, 1944), hemoglobin (Collier, 1947), and hematocrit value by the standard method.

In studies involving nitrogen excretion two rats were maintained in each metabolism cage for the last 8 to 11 days of the exeprimental periods. Excreta were collected at 48-hour intervals, toluene being the urinary preservative employed, and analyzed for total nitrogen by a macro-Kjeldahl method. After the removal of certain organs the remaining carcasses were frozen in liquid air, passed through a power grinder, and a representative sample homogenized in a Waring Blendor. Aliquots were taken for determinations of total nitrogen, of moisture by drying in an electric oven at 105° C. for 5 hours, and of total crude fatty acids (Gavin and McHenry, 1940). Homogenized pooled livers were analyzed for water and protein and also for total vitamin B_6 content (Atkin, Schultz, Williams and Frey, 1943).

Experiment I.—Administration of Anterior Pituitary Growth Hormone to Pyridoxine-deficient and Pyridoxine-supplemented Adult Female Rats.

Sixteen groups, each of 7 female rats with an average initial body weight of 170 gm., were fed the fat-free, 15% casein diet for 25 days before the growth hormone treatments were begun with 8 groups, the other 8 groups not receiving the hormone. Following 7 days of injections the animals were fasted for 16 hours, doses of alanine administered and the levels of blood metabolites determined at intervals of 0, 1, 3 and 6 hours.

Experiment II.—Administration of Anterior Pituitary Growth Hormone to Pyridoxine-deficient and Pyridoxine-supplemented Rats Fed *ad libitum.*

Groups of 8 rats each, of both sexes, with an average initial body weight of 100 gm. were fed the 20% corn oil, 20% casein diet. As a consequence of some mortality those 12 groups which were not used for studies of nitrogen retention were rearranged into 8 groups. After 19 days on diet growth hormone was injected for the next 7 days. Alanine was administered after an 18-hour fast and blood metabolites were measured at intervals of 0, 1½ and 4 hours.

Experiment III.—Administration of Anterior Pituitary Growth Hormone to Pyridoxine-deficient and Pair-fed Control Rats.

Four groups of 8 rats each with an average initial body weight of 89 gm. received the same diet and treatments as those of experiment II except that each control group was pair-fed with its correspoding deficient group. Also, no alanine was given in this experiment. The animals were on diet 15 days before the injections of growth hormone were begun and 10 days afterwards.

RESULTS AND DISCUSSION

During the periods that the rats were on experimental diets the greatest differences in the average body weight of groups were observed in experiment II where the rats were smaller than the females used in experiment I and were fed *ad libitum* unlike those of experiment III. In the last experiment the restriction in food intake prevented the typical effect of growth hormone on body weight. This result would be anticipated since an increase in weight can occur only when quantities of nutrients in excess of maintenance requirements are available for storage. Lee and Schaffer (1934) reported that paired-feeding of growth hormone treated rats and uninjected controls does not prevent an increase in body weight consequent to hormone administration. It should be noted that the average food intakes of those animals (about 12 gm. per day) were far in excess of the average food intakes of the animals in experiment III (6 gm. per day). The administration of growth hormone in all experiments increased the severity of acrodynia, the dermatitis characteristic of pyridoxine deficiency.

TABLE 1. AVERAGE BODY WEIGHT OF EXPERIMENTAL ANIMALS

Exp.	Group	Final average body weight	Weight gains during injection period
		gm.	gm.
I	$+B_6$	197	3
	$-B_6$	165	1
	$+B_6+GH$[1]	207	13
	$-B_6+GH$	166	2
II	$+B_6$	179	7
	$-B_6$	109	2
	$+B_6+GH$	189	17
	$-B_6+GH$	102	−9
III	$+B_6$	141	15
	$-B_6$	104	8
	$+B_6+GH$	140	17
	$-B_6+GH$	100	6

[1] GH indicates groups injected with growth hormone.

From the blood analyses it is evident that in the adult female rats, growth hormone had no effect on the typical picture of urea formation from alanine (Beaton *et al.*, 1950) other than to depress the level of blood urea following alanine administration in both control and deficient groups. The levels of the other blood metabolites indicated that the hormone accentuated the effects of the vitamin deficiency. In the studies with the younger rats, experiments II and III, the fasting level of blood urea was elevated in pyridoxine deficiency as was shown previously by Beaton *et al.* (1950). The effect of growth hormone was apparently to raise it further in the deficient and to depress it in the control animals. Changes in the levels

TABLE 2. BLOOD ANALYSES

Exp.	Group	Hours after alanine load	Blood					Plasma	
			Packed cell volume	Hemoglobin	Amino nitrogen	Urea	Sugar	Glutamic acid	Glutamine
			%	gm.%	mg.%	mg.%	mg.%	mg.%	mg.%
I	$+B_6$	0	46	—	12.6	23	183	1.7	13.5
		1	58	—	36.0	43	207	4.3	—
		3	56	—	15.4	80	165	4.1	—
		6	55	—	12.8	116	156	4.2	—
	$-B_6$	0	44	—	12.8	26	141	3.0	12.5
		1	59	—	44.0	40	185	9.0	—
		3	56	—	17.5	83	229	3.3	—
		6	58	—	15.0	121	156	2.8	—
	$+B_6+GH$	0	43	—	14.2	31	141	2.7	11.0
		1	56	—	34.2	38	207	4.2	—
		3	56	—	15.6	69	223	3.0	—
		6	54	—	12.6	94	177	3.3	—
	$-B_6+GH$	0	44	—	14.0	28	177	2.2	9.5
		1	57	—	50.0	35	163	10.7	—
		3	56	—	20.8	62	255	6.3	—
		6	54	—	14.6	101	129	4.6	—
II	$+B_6$	0	51	15.0	10.3	22	75	5.0	5.5
		1½	51	14.7	45.0	44	190	19.4	—
		4	51	13.9	23.5	90	222	8.3	—
	$-B_6$	0	49	14.4	10.5	32	135	4.6	8.5
		1½	49	14.7	46.2	71	88	13.5	—
		4	48	12.7	23.8	130	247	11.0	—
	$+B_6+GH$	0	49	14.6	13.4	19	84	4.4	6.8
		1½	53	14.7	48.6	30	138	10.4	20.2
		4	50	14.7	23.8	88	230	6.3	—
	$-B_6+GH$	0	46	13.3	11.3	36	160	6.9	14.3
		1½	44	12.7	42.3	65	135	14.3	13.3
		4	48	13.9	14.7	136	215	6.9	—
III	$+B_6$	—	43	13.5	12.9	31	145	3.4	5.0
	$-B_6$	—	42	12.7	12.0	44	105	3.6	4.2
	$+B_6+GH$	—	44	12.3	28.2	25	140	2.2	8.0
	$-B_6+GH$	—	45	12.9	13.0	51	160	4.9	9.3

of plasma glutamic acid and glutamine showed no consistency as a consequence of growth hormone administration.

In control animals growth hormone induced nitrogen retention in the rats fed *ad libitum* but not in those with a restricted food intake. Such a finding was in accord with observations on body weight. Although differences in nitrogen retention and body weight were not seen in the pair-fed animals, growth hormone did affect the levels of blood metabolites. In the absence of pyridoxine, the anabolic properties of the hormone were at no time exhibited.

Analyses for moisture, protein and total crude fatty acids of the body showed that administration of growth hormone had no significant effect on the proportions of water, protein, or of fat in either control or deficient rats.

TABLE 3. NITROGEN EXCRETION

Exp.	Group	No. of rats	No. of days injected	$\frac{\text{Urinary N}}{\text{Food N}}$	$\frac{\text{Fecal N}}{\text{Food N}}$	food N − (Urinary + fecal N) gm./rat/day
II	$+B_6$	8	4, 5	.33	.050	.15
		8	6, 7	.49	.050	.11
	$-B_6$	8	4, 5	.47	.042	.09
		8	6, 7	.50	.057	.08
	$+B_6+GH$	8	4, 5	.22	.035	.27
		8	6, 7	.24	.041	.22
	$-B_6+GH$	8	4, 5	.38	.039	.12
		7	6, 7	.59	.084	.05
III	$+B_6$	8	7, 8	.44	.037	.14
		8	9, 10	.62	.036	.07
	$-B_6$	8	7, 8	.74	.065	.05
		7	9, 10	.77	.055	.03
	$+B_6+GH$	8	7, 8	.48	.045	.10
		8	9, 10	.47	.028	.10
	$+B_6+GH$	8	7, 8	.83	.066	.02
		6	9, 10	.69	.057	.06

TABLE 4. LIVER AND CARCASS CONSTITUENTS FOR EXPERIMENT III

Group	Liver		Carcass		
	% water	% protein	% water	% protein	% crude fatty acids
$+B_6$	73.0	19.8	66.1	23.3	9.6
$-B_6$	73.5	18.7	69.2	24.1	4.0
$+B_6+GH$	70.4	20.2	65.6	21.9	9.6
$-B_6+GH$	78.1	19.1	72.0	25.4	2.9

From the assay of total vitamin B_6 in the liver it is seen that its content was lowered by pyridoxine deficiency per se, and again by growth hormone administration in both control and deficient groups, another finding which suggests that the hormone aggravates the state of the deficiency.

In pyridoxine deficiency the absolute and relative weight of the thymus was greatly reduced. Administration of growth hormone appeared to further that effect. The weight of the adrenal glands increased in proportion to the total body weight in the animals lacking pyridoxine. Again in this respect, growth hormone made the effect more pronounced.

TABLE 5. LIVER TOTAL VITAMIN B_6 CONTENTS AND ORGAN WEIGHTS

Exp.	Group	Total vitamin B_6 of liver µg./gm. moist tissue	Liver % body weight	Spleen % body weight	Thymus % body weight	Adrenal gland % body weight
I	$+B_6$	8.3				
	$-B_6$	6.2				
	$+B_6+GH$	8.4				
	$-B_6+GH$	4.7				
II	$+B_6$	7.0	7.7	0.53	0.37	.008
	$-B_6$	5.4	3.4	0.49	0.07	.011
	$+B_6+GH$	6.7	7.8	0.46	0.31	.011
	$-B_6+GH$	4.8	7.7	0.63	0.07	.018
III	$+B_6$	5.7	5.3	0.57	0.31	.014
	$-B_6$	2.9	5.7	0.48	0.11	.015
	$+B_6+GH$	4.7	5.7	0.69	0.24	.016
	$-B_6+GH$	3.0	5.8	0.36	0.07	.022

In studies of vitamin deficiencies the specificity of effects is always open to question. In most deficiency states there is a decrease in food consumption which gives rise to inanition. The use of pair feeding makes possible comparison between two groups with one variable: presence or absence of a particular vitamin. Observations made on this basis have been reported and discussed above. It should be made clear, however, that the deficiency of only one vitamin has been studied. This one was selected because of the reported relation to protein metabolism. A deficiency of another B vitamin might have given similar results. Until further investigations are made it will not be possible to state that the reported effects are specific for a single vitamin.

SUMMARY

1. Experiments have been described in which pyridoxine deficiency and anterior pituitary growth hormone administration were conjointly studied in adult female rats and in younger rats fed *ad libitum* and on a restricted food intake.

2. The only observed effects of growth hormone on the blood chemistry of pyridoxine-deficient animals following a test-load of alanine were apparent in adult female rats.

3. Provided that the animals were receiving an adequate amount of food, growth hormone promoted increased body weight and nitrogen retention in the control animals but not in the deficient ones.

4. The hormone aggravated the effects of deficiency as demonstrated by the increased severity of acrodynia, the further elevation in the fasting level of blood urea, the lowered content of total vitamin B_6 in the liver, the decreased weight of the thymus, and the increased weight of the adrenal glands. These effects of growth hormone administration on organ weights and on total vitamin B_6 content were also observed in animals provided with pyridoxine.

Acknowledgments

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THE EFFECT OF ASPIRIN UPON THE GLYCOSURIA OF PARTIALLY DEPANCREATIZED RATS IN THE PRESENCE AND ABSENCE OF THE ADRENAL GLANDS

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SALICYLATES and related compounds are capable of activating the adrenal cortices via the anterior pituitary (Cronheim, King and Hyder, 1952). Hailman (1952) has summarized the evidence that the therapeutic and biologic effects of these compounds are mediated by the increased secretory activity of the adrenal cortices.

We have considered the possibility that aspirin can cause a state of hypercorticalism in the laboratory rat. If this were true, it would be expected that aspirin would exacerbate the diabetes of the partially depancreatized rat since corticotropin and the 11-oxygenated steroids of the adrenal cortex are diabetogenic (Ingle, 1948). On the contrary, large doses of aspirin suppress the glycosuria and hyperglycemia of diabetic patients and animals (see Gross and Greenberg, 1948, for review).

In the present study it is shown that large doses of aspirin ameliorate the diabetes of partially depancreatized rats in both the presence and absence of the adrenal glands.

METHODS

Infection-free male rats of the Sprague-Dawley strain were partially depancreatized at a weight of approximately 275 gms. by the procedure described by Ingle and Griffith (1942). The experiments were conducted in an air-conditioned laboratory with the temperature maintained at 74 to 78° F. and the humidity at approximately 50 per cent of saturation. After the animals had recovered from the operation and had reached a weight of approximately 300 gms., they were placed in metabolism cages and were adapted to the force-feeding of a medium carbohydrate diet (Table 1) by stomach tube each morning and late afternoon. The technique and diet were modified from those described by Reinecke, Ball and Samuels (1939). During the period of adaptation to force-feeding the amount of diet was increased gradually to prevent the development of food shock. The animals were brought to a full feeding of 26 cc. of diet per day on the 6th day. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with toluene. Urine glucose was determined by the ferricyanide electrode method of Shaffer and Williams (1935).

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TABLE 1. MEDIUM CARBOHYDRATE DIET

Constituent	grams
Cellu flour (Chicago Dietetic Supply House)	60
Osborne & Mendel salt mixture	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vitamin K (2-methyl-1,4-naphthoquinone)	100 mg.
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc.

After the level of glycosuria had become stable, 50 per cent of the animals were adrenalectomized by sterile technique. The amount of adrenal cortical extract required to restore the glycosuria to its preadrenalectomy level (3 to 5 cc. in divided doses per day) was established for each adrenalectomized rat and was then kept uniform throughout the remainder of the experiment. The adrenal cortical extract (Upjohn) was prepared from mixed beef and hog glands and represents the activity equivalent of 0.1 mg. of hydrocortisone per cc.

Aspirin was ground into a fine suspension in peanut oil at a concentration of 100 mg. per cc. It was administered by subcutaneous injection immediately after the morning and late afternoon feedings.

EXPERIMENTS AND RESULTS

Following control periods of several weeks, each rat was treated with 40 mg. of aspirin daily for 7 days, 80 mg. daily for 7 days and 160 mg. daily for 7 days. When the injections were stopped each animal was observed during a second control period of 14 days. The experiments involved 10 severely diabetic rats, 5 of which were adrenalectomized; 13 moderately diabetic rats, 6 of which were adrenalectomized; and 10 mildly diabetic rats, 5 of which were adrenalectomized.

During the administration of aspirin there was a suppression of the glycosuria in each animal to an extent which was proportional to the dose. When the injections of aspirin were stopped, there was exacerbation of the glycosuria to levels much higher than the initial control values. The pattern and extent of response was similar in the adrenalectomized and non-adrenalectomized animals. The data are summarized in Figure 1.

DISCUSSION

The effect of aspirin upon glycosuria in the rat occurs independently of any change in adrenal function. Smith (1952) has shown that sodium salicylate reduces the glycosuria of steroid (cortisone) diabetes in the rat and also suppresses the glycogenic activity of cortisone.

Other biologic effects of salicylates occur independently of the adrenal cortices. Thorn (1951) has noted the usefulness of aspirin in controlling the fever of patients having adrenal cortical insufficiency.

It would be of special interest to know whether or not the therapeutic effect of aspirin and similar compounds upon the rheumatoid diseases is mediated by the adrenal cortices. This hypothesis has found favor among a number of laboratory and clinical investigators but the supporting evidence is indirect and can be otherwise interpreted. A critical trial of the hypothesis would require the testing of these compounds in patients without functional adrenal glands who also have one of the rheumatoid diseases.

SUMMARY

This study was a partial test of the hypothesis that large doses of aspirin can cause a state of hypercorticalism in the rat. Thirty-three partially depancreatized male rats of about 300 gms. weight were force-fed a medium

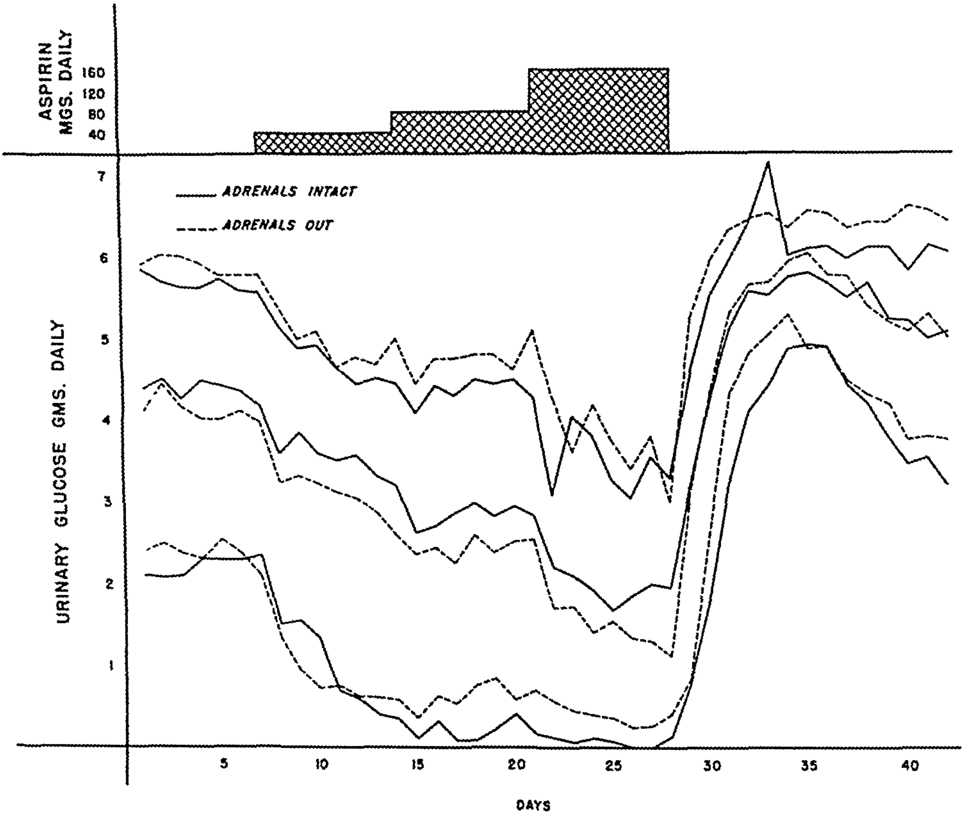

FIG. 1. The effect of aspirin upon the glycosuria of partially depancreatized rats, with and without adrenal glands. Averages.

carbohydrate diet. Sixteen rats of this group were also adrenalectomized and treated with adrenal cortical extract in amounts (3 to 5 cc. daily) which sustained the preadrenalectomy level of glycosuria. Each rat was given subcutaneous injections of aspirin in oil suspension in amounts of 40, 80 and 160 mg. per rat for 7 days per dose. There was a marked suppression of glycosuria to an extent which was related to the size of the dose of aspirin. When the injections of the drug were stopped there was a temporary exacerbation of the glycosuria to much higher levels than were noted during the pre-injection period. Since the pattern and extent of response to aspirin was identical in both the presence and absence of the adrenal glands, it was concluded that this pharmacologic effect of aspirin is not mediated by the adrenal glands.

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ANTAGONISTS TO CORTISONE: AN ACTH-LIKE ACTION OF STEROIDS

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THE experiments reported here were undertaken to determine if some of the effects of cortisone in rats could be antagonized or otherwise modified by the simultaneous administration of other steroids. In the course of the initial studies it was observed that both 17α-methyl-5-androstene-3β,17β-diol, subsequently referred to as MAD, and testosterone propionate (TP) counteracted markedly the adrenal-inhibiting effects of cortisone (Gaunt, Howell and Antonchak, 1952) and subsequent work has been focused largely on that point. By coincidence Winter and Hollings (1951) and Winter, Hollings and Stebbins (1953) independently but earlier observed the same adrenal-maintaining effect of the same two compounds. They put their data at our disposal prior to its publication. We have extended their work particularly by studying the effects of a large number of steroids but in all instances in which duplication occurred we have confirmed their findings.

It has been known since the original work of Cutuly, Cutuly and McCullagh (1938), confirmed by Leonard (1944) and Zizine, Simpson and Evans (1950), that testosterone propionate and some other steroids (Leathem and Brent, 1943; Leonard, 1944) would prevent partially the expected adrenal atrophy after hypophysectomy. Leathem (1944) saw the same effect of testosterone propionate but found that it was a time-limited response which faded out despite continued treatment. We have found that cortisone-induced adrenal atrophy in intact animals can be prevented by testosterone and a large number of other C-19 and C-20, but not C-21, steroids and that this effect cannot be correlated with their androgenic, growth-stimulating or other observed biological properties.

METHODS

Male rats from our inbred Wistar stock weighing approximately 60 grams were used. They were maintained on a commercial laboratory chow supplemented by lettuce and cod liver oil.

Cortisone acetate was given in a dose of 1 mg. per day and other steroids were used in varying dosages. All injections were made subcutaneously. In the initial experiments

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treatments were continued for 20 days. Later it was observed that essentially the same effects could be observed after only seven days of treatment and subsequent experiments were of that duration. Fresh wet weight of organs are recorded.

Since data were too voluminous for all of them to be included in this paper relative organ weights only are tabulated except in Table 5 and 6. Actual weights can easily be estimated from the body weight data. The statistical value included is the standard error$=\sqrt{\sum d^2/n(n-1)}$. Statements that differences between means are definitely significant are based upon p values of less than 0.05 from Fisher's table of t.

Seminal vesicle weights were included as a rough index of the androgenicity of the compounds studied. Since non-castrated animals were used, these figures should not be construed as precise measures of androgenicity.

Cortisone acetate, referred to generally in this paper as cortisone, was given in aqueous suspension. DCA, estradiol benzoate and the 0.25 mg. doses of TP (Tables 1, 2 and 3) were given in oil solution. MAD was administered in an emulsion in the cases recorded in Tables 1, 2 and 3. All the compounds shown in Tables 4 and 6 were injected as microcrystalline aqueous suspensions. No differences attributable to the vehicle were observed.

The adrenal glands were sectioned and examined particularly for changes in lipid content and distribution. One adrenal was fixed in 10 per cent neutral formalin for 48 hours, embedded in gelatin (Zwemer, 1933) and returned to formalin until sectioned. These glands were sectioned at 15 microns on a freezing microtome, stained with sudan IV (Herxheimer's solution, Cowdry, 1943) or with sudan black B and mounted in glychogel (Zwemer, 1933). Some sections were placed in 2.5 per cent ferric ammonium sulfate at 37° C. for three days and then treated with sulfuric acid (Schultz, 1924) in an attempt to reveal cholesterol and its esters. The other adrenal was either fixed in Bouin's fluid or in cold 80 per cent alcohol. The glands fixed in Bouin's were serially sectioned and stained with hematoxylin and eosin. The glands fixed in alcohol were used to estimate the alkaline phosphatase enzyme by the Gomori (1939, 1941) technique with the addition of 2 per cent magnesium sulfate to augment the stain. Such tissues were incubated at 37° C. with sodium beta glycerophosphate for three hours at pH 9.3–9.4.

RESULTS

Adrenal Maintenance

As noted in all of the tables, cortisone when given alone produced the expected adrenal inhibition. This effect was almost as great after 7 as after 20 days of treatment. In hypophysectomized rats cortisone was without effect on the actual adrenal weights but, because of body weight loss, increased the relative adrenal weights.

DCA also reduced adrenal weight (Table 1) but less so than cortisone. When DCA and cortisone were combined their effects in reducing adrenal weight were greater than that of either compound alone in the 20-day experiments (Table 1) but not in those run for seven days (Table 3).

A sodium chloride drinking solution was without effect on adrenal weight when given alone or with cortisone (Table 1). When given with DCA it increased adrenal weight over that seen with DCA alone (Table 1). This may be a manifestation of the expected damaging effects of this combination.

TABLE 1. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN INTACT AND ADRENALECTOMIZED MALE RATS[1]

Experiments of 20 days duration

Group No. & treatment	Body weight—gm.			Organ weight (per 100 gm. body weight)					No. rats
	Initial	Terminal	% Gain	Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testis, gm.	Seminal vesicle, mg.	
Intact Rats									
1. None	65	157	144± 6.0	16.6±0.50	313±15.3	0.91±0.016	1.14±0.023	45.3±2.9	12
2. Cortisone Acetate 1 mg./day	68	121	77± 6.4	7.7±0.55	128±11.9	1.03±0.039	1.42±0.063	53.0±7.8	12
3. DCA 1 mg./day	65	172	165± 9.2	12.9±0.42	304±10.8	1.03±0.013	1.08±0.039	49.0±3.15	12
4. 1% NaCl to drink	63	148	133±10.4	18.2±0.93	299±10.9	0.99±0.010	1.15±0.045	39.5±3.65	12
5. Cortisone Acetate + DCA	64	131	104± 7.7	5.9±0.25	130±21.8	1.19±0.023	1.25±0.051	43.4±3.74	12
6. DCA+NaCl	66	147	123± 9.1	15.3±0.92	270±22.2	1.23±0.035	1.14±0.041	39.4±3.88	12
7. Cortisone Acetate +NaCl	67	109	63± 6.8	6.8±0.42	125±11.6	1.11±0.026	1.48±0.047	56.2±6.65	12
Adrenalectomized Rats									
8. None	All died before 20th day								12
9. Cortisone Acetate 1 mg./day	62	90	47± 5.6	—	107	1.17	1.59	38.2	12
10. DCA—1 mg./day	62	162	164± 6.7	—	408	1.08	1.09	46.8	12
11. 1% NaCl to drink	69	132	90± 6.9	—	439	1.05	1.18	45.2	7
12. Cortisone Acetate +DCA	62	120	96± 6.1	—	100	1.17	1.32	40.5	12
13. DCA+NaCl	66	139	113± 8.6	—	352	1.16	1.12	45.3	10
14. Cortisone Acetate +NaCl	65	110	71± 6.7	—	101	1.11	1.51	63.3	12
Intact Rats									
15. None	58	152	162± 7.0	19.3±1.00	302±15.8	0.94±0.018	1.17±0.030	45± 2.82	13
16. MAD* 2 mg./day	58	147	155± 6.1	19.9±0.45	179±12.0	1.04±0.015	0.89±0.04	307±17.00	12
17. Cortisone Acetate +MAD	57	96	68± 4.1	22.3±1.29	66.7± 6.5	1.15±0.026	1.26±0.063	460±27.82	12
18. Cortisone Acetate +DCA+MAD	59	117	100± 6.6	20.5±1.21	68.0±11.4	1.19±0.026	1.11±0.023	468±27.55	12
19. Testosterone Propionate 0.25 mg./day	60	158	161±10.9	18.7±1.40	167±13.3	0.97±0.010	0.47±0.028	398±40.85	12
20. Cortisone Acetate +Test. Prop.	59	122	106± 7.3	11.7±0.53	87± 6.7	0.98±0.022	0.79±0.041	484±44.82	12
21. Cortisone Acetate+Test. *Prop.*+DCA	60	133	124± 7.9	10.3±0.24	74± 6.0	1.1 ±0.017	0.65±0.011	511±34.27	12

* MAD = 17α-methyl-5-androstene-3β,17β-diol.

[1] In all cases in this and subsequent tables when combined treatments were used the dose of each was the same as that indicated when the substances were given separately.

The first androgens studied were MAD and TP. They were given initially in daily doses of 2 and 0.25 mgs. respectively in order to achieve approximate equality of androgenicity as judged by the effects on seminal vesicel weight. In such dosage neither MAD nor TP given alone had significant effect on the adrenals of intact animals. Both compounds, however, when given with cortisone helped prevent adrenal atrophy, with MAD being the more effective of the two (Tables 1 and 3). When given in equal doses (Tables 4 and 5) they had equal effects and maintained normal relative adrenal weights and near-normal absolute weights in cortisone-treated rats.

In hypophysectomized rats (Table 2) TP at a 0.25 mg. daily dosage was without effect on adrenal size. MAD (2 mg. per day), on the other hand,

TABLE 2. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN HYPOPHYSECTOMIZED MALE RATS
Experiments of 20 days duration

Group No. & treatment	Body weight—gm.			Organ weights (per 100 gm. body weight)					No. rats
	Initial.	Terminal	% gain	Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testis, gm.	Seminal vesicle, mg.	
1. Hypx.—none	86	91	+ 5.2	11.7±0.42	228	0.72	0.30	8.2	16
2. Cortisone Acetate 1 mg./day	83	68	−18.1	14.6±0.32	51	0.94	0.31	10.3	19
3. MAD 2 mg./day	85	91	+ 7.0	16.7±0.84	55	0.85	1.02	1025.0	20
4. MAD +Cortisone Acetate	86	71	−17.2	21.6±0.17	41	1.03	1.40	1140.0	20
5. Test. Prop. 0.25 mg./day	88	100	+13.7	12.2±0.74	113	0.73	0.57	1010.0	8
6. Test. Prop. +Cortisone Acetate	85	78	−9.1	18.0±0.87	58	0.95	0.98	1472.0	10

produced a distinct increase in adrenal weight. When cortisone was given in combination with MAD a normal relative adrenal weight was obtained. Actual weights, however, were no greater than when MAD alone was given.

TABLE 3. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN INTACT MALE RATS
Experiments of 7 days duration

Group No. & treatment	Body weight—gm.			Food intake/rat/day, gm.	Organ weights (per 100 gm. body weight)					No. rat
	Initial	Terminal	% gain		Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testis, gm.	Seminal vesicle, mg.	
1. None	64	99	55±1.90	11.0	20.6± .64	350	1.05	.843	14.9	12
2. Cortisone Acetate 1 mg./day	63	70	11±1.40	8.9	11.0± .63	72	1.21	1.044	16.7	12
3. Cortisone Ac.+DCA 1 mg./day	64	81	27±2.83	9.7	10.7± .51	90	1.18	.951	14.4	12
4. Test. Prop. 0.25 mg./day	63	103	62±2.19	11.4	19.5± .68	270	1.09	.618	127.8	12
5. Test. Prop.+Cortisone Ac.	59	78	22±2.30	10.4	15.0± .81	67	1.20	.773	181.1	12
6. Test. Prop+Cortisone Ac.+DCA	56	74	33±2.29	9.3	15.9± .97	61	1.19	.779	182.3	12
7. MAD 2 mg./day	60	93	54±4.43	10.3	22.6±1.53	315	1.057	.723	59.4	12
8. MAD+Cortisone Ac.	60	71	18±2.55	8.9	19.2± .67	56	1.178	.847	84.3	12
9. MAD+Cortisone Ac.+DCA	58	75	31±2.60	9.1	20.0±1.52	67	1.241	.830	92.6	12
10. Estradiol Benzoate 10γ/day	58	82	42±2.92	8.6	23.3± .77	223	1.056	.357	30.1	12
11. Estradiol Benz. +Cortisone Ac.	59	72	20±2.14	8.0	17.4±1.30	84	1.032	.456	36.4	12

It is apparent from Tables 4 and 5 that a sizeable group of other steroids are as effective as MAD or TP in preventing cortisone inhibition of the adrenals. None of these compounds, when given alone, increased adrenal size significantly when compared with control groups observed simultaneously with the possible exception of 17β-hydroxyandrostan-3-one. Some of them may have been expected to depress adrenal weight if given for long periods (Selye and Stone, 1950). Most of the C-19 and C-20 compounds were of approximately equal effectiveness. The 21-carbon ethy, and vinyl derivatives of androstenediol or testosterone were, however, curiously weak or ineffective.

TABLE 4. EFFECT OF VARIOUS STEROIDS ON GROWTH AND RELATIVE ORGAN WEIGHTS IN INTACT MALE RATS

Experiments of 7 days duration
(Daily dosage: Cortisone 1 mg.; All others 2 mg.;—10 rats per group)

Group No. & treatment	Body weight		% gain	Food intake/rat/day, gm.	Organ weights (100 gm. body weight)				
	Initial	Terminal			Adrenal, mg.	Thymus, mg.	Kidney, gm.	Testes, gm.	Seminal vesicle
1. None	62	97	57±2.0	14.3	20.1±0.56	336	1.13	0.79	14.4
2. *None	58	95	64±3.4	14.3	22.4±0.77	479	1.17	0.70	12.8
3. Cortisone	63	68	12±2.4	11.6	10.3±0.65	63	1.28	1.01	15.8
4. *Cortisone	59	68	15±2.3	12.2	12.3±0.69	47	1.41	0.92	15.8
5. 17β-hydroxyandrostan-3-one	59	94	57±2.8	13.9	23.6±1.01	208	1.13	0.71	164.0
No. 5+Cortisone	61	75	20±2.9	12.0	23.4±1.35	49	1.38	0.89	233.0
6. *17α-methyl-17β-hydroxy-androstan-3-one	62	105	70±3.4	16.0	23.0±1.17	213	1.22	0.56	149.8
No. 6+Cortisone	62	80	31±3.9	14.7	23.3±0.83	50	1.36	0.70	202.0
7. 17α-Methyl-5-androstene-3β, 17β-diol	61	100	60±2.7	14.3	23.6±1.03	224	1.13	0.74	133.0
No. 7+Cortisone	62	77	22±2.6	12.4	22.3±1.02	52	1.30	0.91	174.0
8. 17α-Methyltestosterone	58	93	58±3.8	11.6	23.5±0.90	226	1.09	0.71	166.0
No. 8+Cortisone	59	75	25±2.8	10.0	21.6±0.76	44	1.27	0.90	212.0
9. *17β-methoxy-4-androsten-3-one	60	102	71±1.5	15.2	21.9±6.70	344	1.19	0.65	71.8
No. 9+Cortisone	61	75	24±2.3	13.2	21.5±0.49	60	1.30	0.85	110.3
10. *Testosterone Propionate	58	102	75±3.3	14.6	22.9±0.70	204	1.18	0.67	179.0
No. 10+Cortisone	59	76	30±2.7	13.1	20.8±1.03	49	1.50	0.83	234.0
11. 5-androstene-3β,17β-diol	58	95	59±4.3	13.4	21.2±1.14	299	1.13	0.68	48.2
No. 11+Cortisone	57	70	20±3.0	11.2	20.7±1.51	58	1.26	0.94	68.4
12. 17α-methyl-3β,17β-androstanediol	57	94	62±4.3	13.6	21.7±1.30	214	1.12	0.73	75.2
No. 12+Cortisone	59	72	20±2.3	12.9	20.7±1.22	49	1.26	0.73	105.5
13. Androstane-3α,17β-diol	57	92	60±3.6	13.0	23.3±1.02	213	1.17	0.70	171.8
No. 13+Cortisone	57	71	23±2.0	11.3	19.9±1.33	46	1.32	0.88	213.5
14. 17α-Methylandrostan-17β-ol	62	98	57±3.3	14.1	20.2±0.86	296	1.10	0.54	45.3
No. 14+Cortisone	60	71	18±2.5	11.7	18.0±0.78	49	1.27	0.72	61.3
15. *Dehydroepiandrosterone	62	104	68±2.1	15.9	17.6±0.87	358	1.16	0.73	29.0
No. 15+Cortisone	62	76	23±2.9	13.7	16.1±0.65	74	1.50	0.92	33.6
16. *17α-Ethylandrostane-diol	59	94	60±3.2	14.6	21.9±1.00	368	1.18	0.70	12.7
No. 16+Cortisone	61	71	18±2.4	12.6	15.4±0.48	80	1.31	0.83	17.3
17. 17α-Ethyltestosterone	60	93	62±5.0	13.9	18.8±1.3	287	1.04	0.53	91.6
No. 17+Cortisone	61	73	20±3.0	12.6	14.0±0.85	52	1.22	0.67	127.4
18. 17α-Vinyltestosterone	63	98	58±3.0	13.0	18.9±0.64	309	1.16	0.52	93.1
No. 18+Cortisone	60	74	22±2.9	13.5	12.8±0.77	62	1.22	0.62	129.3
19. 17α-Ethyl-5-androstene-3β, 17β-diol	60	95	56±5.5	13.4	20.8±1.62	323	1.10	0.79	24.3
No. 19+Cortisone	61	72	18±2.3	11.8	12.7±0.97	67	1.21	0.97	32.0
20. *3β-Methoxy-5-androsten-17-one	60	98	62±3.2	14.7	21.9±0.52	360	1.15	0.78	14.8
No. 20+Cortisone	60	72	20±3.4	13.1	12.1±1.03	84	1.32	1.05	20.8
21. 17α-Vinyl-5-androstene-3β, 17β-diol	62	101	56±3.3	14.4	19.7±0.85	327	1.12	0.80	21.8
No. 21+Cortisone	56	68	21±3.5	10.8	11.6±0.88	66	1.17	0.90	23.1

* The groups marked by an asterisk were observed in one continuous experiment and those not so designated in another.

It happened that our first observation of adrenal maintenance in cortisone-treated animals was with 2 mg. daily doses of MAD. For the sake of uniformity most other steroids were compared with MAD at that same dose level. It is apparent, however, from a cursory study with the small number of animals shown in Table 6 that at least MAD and 5-androstene-3β,17β-diol were effective in doses of less than 2 mg. per day. The same result was found by Winter *et al.* (1953). It is also apparent (Table

TABLE 5. SUMMARY OF DATA ON EFFECT OF CORTISONE ALONE AND IN COMBINATION WITH OTHER STEROIDS ON ADRENAL WEIGHT

Treatment[1]	Adrenal weight, mg.	Adrenal weight, mg./100 gm.	No. rats
None	20.3	21.0	32
Cortisone Alone	7.7	11.2	33
Cortisone + the following:			
17β-hydroxyandrostan-3-one (dihydrotestosterone)	17.1	23.4	10
17α-methyl-17β-hydroxyandrostan-3-one (methyldihydrotestosterone)	18.8	23.3	10
17α-methyl-5-androstene-3β,17β-diol (MAD)	17.1	22.3	10
17α-methyltestosterone	16.0	21.6	10
17β-methoxy-4-androsten-3-one (Testosterone methyl ether)	16.1	21.5	10
Testosterone Propionate	15.7	20.8	10
5-androstene-3β,17β-diol	14.2	20.7	10
17α-methyl-3β,17β-androstanediol	15.1	20.7	10
Androstane-3α,17β-diol	14.1	19.9	10
17α-Methylandrostan-17β-ol	12.7	18.0	10
Estradiol benzoate—10γ/day	12.5	17.4	12
Dehydroepiandrosterone	12.0	16.1	10
17α-ethylandrostane-3β,17β-diol	10.8	15.4	10
17α-ethyltestosterone	10.0	14.0	10
17α-vinyltestosterone	9.7	12.8	10
17α-Ethyl-5-androstene-3β,17β-diol	9.3	12.7	10
3β-Methoxy-5-androsten-17-one (Dehydroepiandrosterone methyl ether)	8.5	12.1	10
17α-Vinyl-5-androstene-3β,17β-diol	7.8	11.6	10

[1] All dosages were: 1 mg. Cortisone + 2 mg. of other steroids (except Estradiol benzoate) per day for 7 days. The effect of the various steroids given alone, together with other related data, is shown in Tables 3 and 4.

TABLE 6. EFFECT OF 17α-METHYL-5-ANDROSTENE-3β,17β-DIOL (MAD) AND 5-ANDROSTENE-3β,17β-DIOL (AD) ON ADRENAL WEIGHT WHEN GIVEN ORALLY OR IN SMALL SUBCUTANEOUS DOSES

(4 rats per group, treated for 7 days)

Daily treatment	Adrenal weight, mg.	Adrenal weight, mg./100 gm.
2 mg. MAD by stomach tube + 1 mg. Cortisone subcu.	9.1	13.8
2 mg. AD by stomach tube + 1 mg. Cortisone subcu.	8.3	11.9
1 mg. MAD subcu. + 1 mg. Cortisone subcu.	15.2	21.8
1 mg. AD subcu. + 1 mg. Cortisone subcu.	13.6	18.5
0.5 mg. MAD subcu. + 1 mg. Cortisone subcu.	13.4	20.4
0.5 mg. AD subcu. + 1 mg. Cortisone subcu.	10.7	15.7

6) that neither of the two above-mentioned compounds were fully active when given orally in 2 mg. doses. Further studies along this line are in progress.

The effects of small doses of estradiol benzoate were surprisingly like those of the larger doses of androgenic steroids in maintaining adrenal weight in the cortisone-treated animals (Table 3). It is a well-known property of estrogens to cause an adrenal weight increment in intact animals when given alone and that was seen here. Given with cortisone it had an effect on adrenal maintenance intermediate between that of the most active and least active compounds shown in Table 5. Further studies with steroids related to estradiol are now in progress.

Adrenal Histology

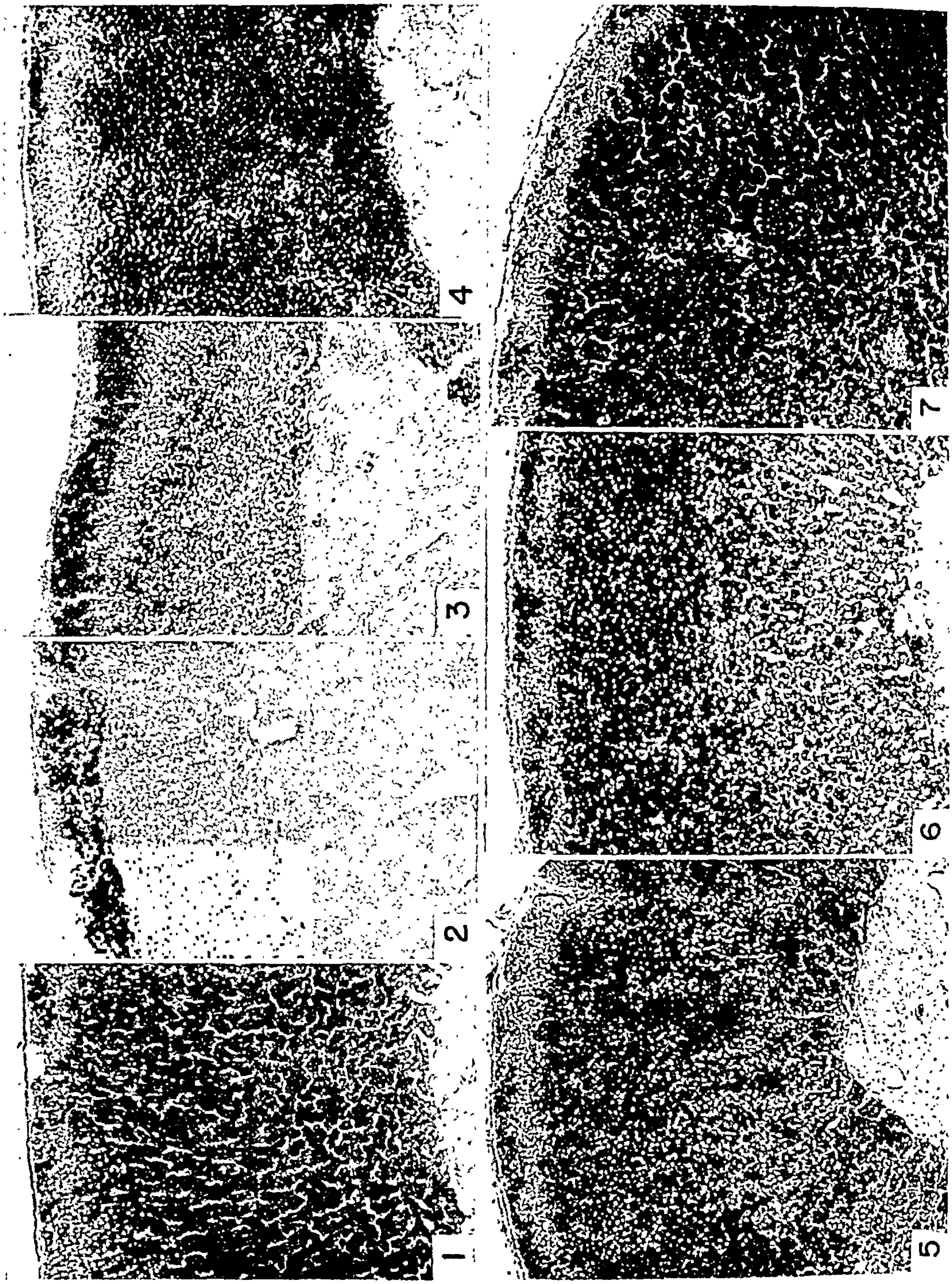
Comprehensive histological study was done only on the adrenals from the animals shown in Table 3 and on those from Table 4 treated with 2 mg. daily of TP and 17β-methoxy-4-androsten-3-one with their appropriate controls. The latter compound was of especial interest because while it maintained adrenal weight as well as TP its androgenic activity was greatly reduced.

a. Lipid: The control glands presented the typical concentration of lipid in the adrenal cortex (Fig. 1). The glomerulosa layers were generally narrow and while most cells contained lipid an occasional cluster lacked it and broke the continuity of a continuous ring under the connective tissue capsule. A sudanophobic zone separated the glomerulosa and fasciculata. This band of lipid free cells apparently is a characteristic of the male rat. The outer fasciculata was fat laden and this heavy concentration in some cases continued to the reticularis or in other cases a gradual decrease in concentration occurred toward and through the reticularis.

Administration of cortisone acetate appeared to favor an enlargement of the glomerulosa emphasized by the intense lipid staining of many of the cells (Fig. 2). An occasional adrenal exhibited some fascicular fat, generally in the outer half, but the staining intensity was always subnormal. In more than half of the glands, the fasciculata and reticularis were lipid free; these tended to be the smaller glands of the series.

The simultaneous administration of 1 mg. per day of DCA with cortisone unexpectedly offered some protection from lipid depletion (Fig. 3). Some fascicular fat was present, ranging from a tinge to a moderate amount but the lipid was always relegated to the outer third or half of the fasciculata. Cortisone seemed to have the dominance in enlarging the glomerulosa but the lipid was not uniformly heavy in that zone.

MAD in 2 mg. daily amounts had no effect on adrenal lipid distribution.



FIGS. 1–7

However, when combined with cortisone the depleting effects of the cortisone were largely prevented and the lipid distribution was within the range of normal limits (Fig. 5). The impression was gained, however, that the glomerulosa was still enlarged by the cortisone but when DCA was added in a triplicate treatment that the glomerulosa returned to normal size in some but not all cases (Fig. 7).

Testosterone proprionate in 0.25 mg. daily amounts whether given alone, with cortisone or with cortisone and DCA had no effects except to inhibit slightly the lipid-depleting effects of cortisone. Two mg. doses of TP and of 17β-methoxy-4-androsten-3-one had effects indistinguishable from those described above with the same dose of MAD (Figs. 4 and 6).

Estradiol benzoate (10γ per day) effectively eliminated the sudanophobic zone (as per Greep and Jones, 1950) but did not alter lipid in other parts of the cortex. When estradiol was given with cortisone the lipid distribution was otherwise normal in four of ten cases. The six remaining cases had enlarged glomerular zones which were fat-laden while other parts of the cortex showed only a uniform hazy film of stain. The pattern was that of a cortisone-treated gland but instead of the fasciculata and reticularis being mostly fat free there was always some lipid.

b. *Alkaline phosphatase:* Using glycerophosphate as a substrate, the normal adrenal exhibited a considerable concentration of enzyme in the glomerulosa; the nuclei stained markedly and the cytoplasm slightly. Only slight amounts were observed in the nuclei and none in the cytoplasm of the other cortical cells. Cortisone appeared to reduce slightly the fascicular enzyme concentration whereas MAD and TP tended to increase it slightly. The uncertain impression was gained that the increase in alkaline phosphatase imposed by androgen was not altered by simultaneous treatment with cortisone.

c. *Cholesterol esters:* The Schultz technique, presumably revealing cholesterol or its esters, was postive in the adrenal cortices of all control rats.

←⋘

FIGS. 1–7. Adrenals stained with sudan black B from animals treated with various steroids for seven days. ×176. These figures are described in the text with other related data shown in tables as indicated below. Lipid content essentially normal in Figures 1 and 4 to 7.

1. Control—no treatment (Table 3).
2. Cortisone acetate only (Table 3).
3. Cortisone+DCA (Table 3).
4. Cortisone+17β-methoxy-4-androsten-3-one (Tables 4 and 5).
5. Cortisone+17α-methyl-5-androstene-3β,17β-diol (Table 3).
6. Cortisone+Testosterone Propionate (Tables 4 and 5).
7. Cortisone+17α-methyl-5-androstene-3β,17β-diol+DCA (Table 3).

pounds which did not antagonize cortisone tended to decrease adrenal weight when given alone in our short-term experiments (Table 4). It is of interest that the "blocking" of an active grouping at position 3 of the steroid molecule by making the 3-methyl ether of dehydroepiandrosterone (No. 20 of Table 4) completely eliminated its adrenal-maintaining ability. This chemical maneuver will be more enlightening when tried on other more active compounds because dehydroepiandrosterone (No. 15 of Table 4) antagonized cortisone only weakly and by itself tended to be an adrenal inhibitor. The "blocking" of position 17 of testosterone by making its 17-methyl ether (No. 9 of Table 4), while greatly reducing its androgenic activity (Table 4 and unpublished observations), did not alter its ability to antagonize cortisone.

Correlation with other Biological Properties

No apparent correlation can be made between other biological properties of the steroids tested and their ability to prevent cortisone inhibition of the adrenals. Most of the compounds used were androgens. Some were strong, however, and some very weak as judged by their ability to stimulate the seminal vesicle and not all of the androgens tried would maintain the adrenals. Furthermore, the positive effects of estradiol benzoate in small doses shows that the effect is not limited to the classical androgens. Most of the non-corticoids used inhibited testes but that was true to variable extents and it occurred whether or not the compounds maintained the adrenals. Likewise all of the compounds used tended to antagonize the body growth-inhibiting effects of cortisone irrespective of their effect on the adrenal. It is likely, therefore, that adrenal maintenance is a separable property of a large group of steroids which at the moment have no other obvious common feature.

Growth

It is a common clinical finding that the administration of androgens with cortisone prevents some of the catabolic effects of the cortisone (e.g. Kinsell, 1951; Clark, Manning and Appleton, 1951; Luft and Sjogren, 1951). It is likely that the ability of testosterone and other steroids as seen here to prevent partially cortisone-induced growth inhibition is a manifestation of what is usually called the anabolic action of steroids. If this be true, the animal whose growth is inhibited by cortisone might serve as an assay specimen for such anabolic steroids. In that case, however, it would have to be said the MAD is a weaker anabolic stimulant than TP when given in doses that are of approximately equal androgenicity. Before such conclusions are made, however, our observations would have to be correlated with nitrogen metabolism in pair-fed or force-fed animals and distinction made between weight increment due to salt and water retention and tissue growth. In any case it is of interest that growth in the cortisone-treated animal is maintained better by a combination of DCA and TP than by either substance alone.

The growth-inhibiton and other toxic manifestations of cortisone or ACTH treatment have been in part antagonized by a variety of means such as liver therapy (Ershoff, 1951), aureomycin (Meites, 1952), potassium (Whitney and Bennett, 1952; Glafkides *et al.*, 1952) and fat (Kinsell *et al.*, 1951). Such results plus those of the type reported here with steroids indicate clearly that the continuing search for agents which ameloriate the unwanted effects of cortisone is worthwhile.

SUMMARY

1. A large number of steroids including methylandrostenediol and testosterone propionate prevented the adrenal-inhibitng effect of cortisone in rats as judged by the maintenance of adrenal weight, general histological appearance and the amount and distribution of sudanophilic lipid. Most of the steroids tried which contained 19 or 20 carbon atoms were effective in preventing adrenal atrophy; C-21 steroids were either weak or inactive. Such results with TP and MAD confirm the findings of Winter *et al.* The effects were observed of several steroids in triplicate combinations and of a high salt diet in combination with DCA and cortisone.

2. The majority of the compounds which prevented cortisone inhibition of the adrenals possessed some androgenic activity but their effectiveness was not correlated with their relative androgenicity and all androgenic compounds did not prevent adrenal atrophy. Estradiol in small doses also had to some degree the ability to antagonize the adrenal-depressing effects of cortisone.

3. Compounds which were effective in preventing cortisone from causing adrenal atrophy had little, if any, adrenal-stimulating effect when given alone.

4. Cortisone was given in a dose of 1 mg. per day and most other steroids, either alone or in combination with cortisone, at a dose of 2 mg. per day. Preliminary experiments, however, suggested that methylandrostendiol and presumably other steroids can prevent cortisone-induced adrenal atrophy at a much smaller daily dose than 2 mg. when injected but that its effectiveness is reduced by oral administration. Estradiol benzoate had some activity in 10γ doses.

5. In hypophysectomized rats methylandrostenediol given alone partially prevented adrenal atrophy. When given with cortisone, relative but not absolute adrenal weights were enhanced. Cortisone alone increased the relative but not absolute adrenal weight after hypophysectomy. The

conclusion is not yet warranted that the adrenal-maintaining ability of combinations of steroids is independent of the pituitary since a given treatment was found to be more effective in intact than in hypophysectomized animals.

6. Cortisone depleted the sudanophilic lipids of the fasciculata and reticularis, an action which was blocked by methylandrostenediol, testosterone propionate and 17β-methoxy-4-androsten-3-one. The latter compounds were without effect on sudanophilia when given alone. Estradiol benzoate eliminated the sudanophobic zone of the male rat adrenal and partially inhibited the lipid-depleting effects of cortisone. More lipid was present in glands of animals treated with cortisone plus DCA than in those given cortisone only. A different pattern of results was obtained, however, when adrenals were stained by the Schultz technique for cholesterol or its esters.

7. Testosterone propionate, DCA and most of the other steroids used, including estradiol benzoate, antagonized to some degree the body weight-inhibiting effects of cortisone whether or not they prevented adrenal atrophy. In some but not all groups, the animals on combined therapies ate more food than those on cortisone alone.

8. The normal weight increment of the testes and the kidneys was inhibited less by cortisone than that of the body as a whole.

9. It is suggested that the ability to prevent cortisone inhibition of the adrenal defines a group of steroids without other common biological properties.

ADDENDUM

Work completed since the foregoing was written has established the following: (1) Judged by its effects on adrenal weight and maintenance of sudanophilic lipids at low dosage (0.25 mg. per day) MAD is the most effective steroid in preventing adrenal atrophy of the several compounds which seemed equally active at high dosage (2 mg. per day) in Table 5. (2) The following were either weak or inactive at 2 mg. daily doses: progesterone, ethinyltestosterone, pregnenolone, 3β-methoxy-5-pregnen-20-one, and 4-androsten-3-one. The 3-methyl ethers of MAD and androstenediol were slightly less active than the latter compounds themselves. Such results, together with those recorded in the above paper, show that some functional group at C-17, best exemplified by a β-hydroxyl and probably enhanced by the presence of an α-methyl group, is necessary for adrenal-maintaining activity. The weakness or inactivity of C-21 compounds is confirmed. Alterations in functional groups at C-3, or their absence, result in quantitative variations of, but not loss of, activity. (3) Further work with estradiol benzoate, α- and β-estradiol and stilbestrol indicates that they exert only a weak and inconstant activity which unlike the more effective steroids is probably due to their stress-like stimulation of the adrenal.

Acknowledgments

We are indebted to our colleagues Dr. A. A. Renzi, Miss Martha Gilman and Miss Gloria Joy Miller for assistance in various phases of this work, to Mr. Benny Antonchak for the histological preparations and to Mr. Robert Wolf for the photography. In particular, however, our thanks are due to the Chemical Research Division, especially Dr. Arthur St. Andre, for preparing all of the steroids not commercially available and to Dr. E. Oppenheimer for his stimulating interest and suggestions.

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ANEMIA IN THE RABBIT DURING PREGNANCY AND FOLLOWING TREATMENT WITH WATER SOLUBLE OVARIAN EXTRACTS[1,2]

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ANEMIA of pregnancy has been described in a number of widely divergent species of mammals. In general it has been concluded that this phenomenon is due to a hemodilution (Bond, 1948; Houssay, 1951), although the etiologic factors responsible for the anemia remain unknown. Some investigators still feel that the anemia may be due to nutritional deficiencies. Forman (1950) has reported excellent results in pregnant women with a molybdenum iron complex, which caused an increase in the hemoglobin and prevented the anemia of pregnancy. However the work of Mitchell and Miller (1931), Beard and Myers (1931) and Van Donk, Feldman and Steenback (1934) on the rat, would seem to indicate that this anemia is not due to a lack of nutritional factors.

The ability of different hormones or the lack of these hormones to cause an anemia has been reported rather extensively. It has been shown that hypophysectomy, throidectomy or treatment with estrogens depress the number of circulating erythrocytes (see review by Gordon and Charipper, 1947). Recently Witten and Bradbury (1951) reported a hemodilution and decrease in the red blood cell count in women after treatment with estrogen. Studies in the rat indicate that the placenta is the source of the factor (or factors) responsible for the anemia of pregnancy (Newcomer, 1947), and that the anemia is due to a hemodilution (Bond, 1948). These results would tend to suggest that the estrogens, which are present in increasing amounts during pregnancy, may be the etiologic agents.

The present investigation was undertaken to elucidate the factors responsible for the anemia of pregnancy. In the initial phase of this study, the normal pattern of pregnancy was followed by determining the changes in the water balance, the circulating erythrocytes, leucotyces, hemoglobin, hematocrit, non-protein nitrogen and chlorides of the blood and the urinary chorides. In the second part of this study the same determinations

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[1] This investigation was started at the Biological Laboratories, Harvard University, Cambridge, Mass.

[2] Aided in part by grants from the Thompson Science Fund, Boston, Massachusetts and the Chilcott Laboratories, The Maltine Co., Morris Plains, New Jersey.

were made in rabbits during treatment with a water soluble extract of the ovary of pregnant sows. This preparation (relaxin) was tested in these experiments since previous work (Zarrow 1949) had shown that it possessed the ability to cause water retention and thus possibly produce a hemodilution.

MATERIAL AND METHODS

Grey, chinchilla rabbits obtained from Rockland Farms were used in the present study. All animals were housed under normal conditions of constant temperature and humidity with free access to Purina rabbit chow, lettuce and water. During the course of the experiments some of the rabbits were placed in metabolism cages and daily measurements made on their water intake and urine output. Blood samples were obtained from the marginal vein of the ear at regular intervals of 5, 10, 15, 20, 25, and 30 days after mating and at 5 and 15 days after parturition. In the experiments involving treatment with ovarian extracts, blood samples were obtained every third day and a minimum of three control samples were obtained prior to mating or treatment.

Total erythrocyte and leucocyte counts were carried out in duplicate and hemoglobin concentrations were determined in a Klett photoelectric colorimeter after dilution with 0.1 N hydrochloric acid. Reticulocyte counts were made using brilliant cresyl blue and a minimum of 1,000 red blood cells were counted. Hematocrit determinations were carried out in Wintrobe tubes after centrifugation for 30 minutes at 2,500 R.P.M. Non-protein nitrogen determinations of the blood were made according to the micro-Kjeldahl method using sulfuric and phosphoric acid for digestion and determining the ammonia colorimetrically after direct Nesslerization (Hawk, Oser and Summerson, 1947). The serum chlorides were determined according to the method of Shales and Shales (1941) by titrating with a standard mercuric nitrate solution and using diphenyl carbazone as an indicator. Urinary chlorides were determined as above except that the urine was first treated with norit and 10 per cent sodium nitro-prusside was used as the indicator.

Several different relaxin preparations[3] were used in this study as the source of the water soluble factor. In all instances the extract was obtained from the ovaries of pregnant sows and purified for its relaxin content. Consequently the amount of ovarian ovarian extract used in these experiments is expressed in guinea pig units (GPU) of relaxin.

OBSERVATIONS AND RESULTS

Blood Constituents During Pregnancy

Data on the hemoglobin and cellular elements of the blood of the normal, pregnant and post partum rabbit may be seen in Table 1. The normal rabbits of this strain possess a red blood cell count of 5,950,000 per cmm. A highly, significant decrease in the circulating erythrocytes is seen by the 20th to the 25th day of pregnancy. The decline is progressive throughout

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[3] Relaxin preparation P XXVII-5 was obtained through the courtesy of Dr. E. H. Frieden and several other relaxin preparations (Releasin) were obtained from Dr. Kroc, Chilcott Laboratories. Preparation J46 was prepared by one of us (MXZ) and has been used in other studies involving the action of relaxin (Zarrow and Money, 1948; Zarrow, 1949).

TABLE 1. CHANGES IN THE CELLULAR COMPONENTS AND HEMOGLOBIN OF THE BLOOD OF THE RABBIT DURING PREGNANCY[1]

	Erythrocytes, million per cmm.	Hb., gm. %	Hematocrit, %	Reticulocytes, %	Color index
Days pregnant					
0	5.95 ± 0.083[2]	11.9 ± 0.22	38.7 ± 0.69	2.1 ± 0.24	1.00[3]
5	5.85 ± 0.085	11.9 ± 0.32	39.0 ± 0.87	2.2 ± 0.36	1.02
10	5.70 ± 0.10	11.8 ± 0.28	38.0 ± 0.84	2.6 ± 0.35	1.04
15	5.43 ± 0.14	11.7 ± 0.21	37.0 ± 1.20	3.9 ± 0.42	1.07
20	5.12 ± 0.14	11.3 ± 0.22	35.3 ± 1.36	6.8 ± 1.03	1.09
25	5.00 ± 0.18	10.8 ± 0.31	33.4 ± 1.45	3.8 ± 0.72	1.07
30	4.71 ± 0.12	9.9 ± 0.34	32.1 ± 0.34	2.4 ± 0.43	1.03
Days post partum					
5	5.09 ± 0.18	10.2 ± 0.40	33.2 ± 0.40	5.3 ± 0.36	1.00
15	5.81 ± 0.088	11.4 ± 0.32	34.3 ± 1.01	2.2 ± 0.16	1.01

[1] Ten rabbits were used and a total of 12 pregnancies studied.

[2] Standard error $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

[3] $\frac{\text{gm. Hb/100 ml. pregnant rabbit blood}}{\text{gm. Hb/100 ml. normal rabbit blood}} \div \frac{\text{RBC count of pregnant rabbit}}{\text{RBC count of normal rabbit}}$

gestation with a return to normal levels after parturition. As early as 5 days postpartum the number of circulating red blood cells has returned to the normal value. The average decline in erythrocytes is approximately of the order of 20 per cent. Along with the drop in red blood cells may be seen a concommitant decline in hemoglobin and hematocrit. All three appear to parallel each other throughout gestation with the most rapid drop occurring between the 20th day and the termination of pregnancy. Complete recovery in the hemoglobin concentration may be noted by the 15th day after delivery. The color index also indicates the parallel nature of the changes in the red blood cell count and hemoglobin concentration. The hematocrit showed a drop of 17% by the 30th day of pregnancy and was still 11% below normal 15 days after parturition.

A rise in reticulocytes may be seen by the 15th day of pregnancy with a peak on the 20th day. In spite of the increasing severity of the anemia the reticulocytes returned to normal just prior to partutition and showed a second rise on the 5th day postpartum.

Determination of the non-protein nitrogen of the blood indicated a marked and progressive drop throughout the course of pregnancy. The first significant change was noted on the 20th day of pregnancy when the concentration decreased from a normal of 42.6 mg. % to 33.4 mg.%. The decline continued throughout pregnancy with partial recovery by the 5th day postpartum and a return to normal by the 15th day. Serum chlorides failed to indicate any significant changes during pregnancy or the postpartum period. The urinary chlorides showed a steady decline in the rate of excretion throughout the course of pregnancy and a return to normal by the 5th day post partum (Table 2). However, it is difficult at this time to state whether the drop was significant due to the high standard errors obtained with the urinary chlorides. Figure 1 shows the entire sequence of events during pregnancy in a normal rabbit and indicates the correlation in time for changes in the various blood components.

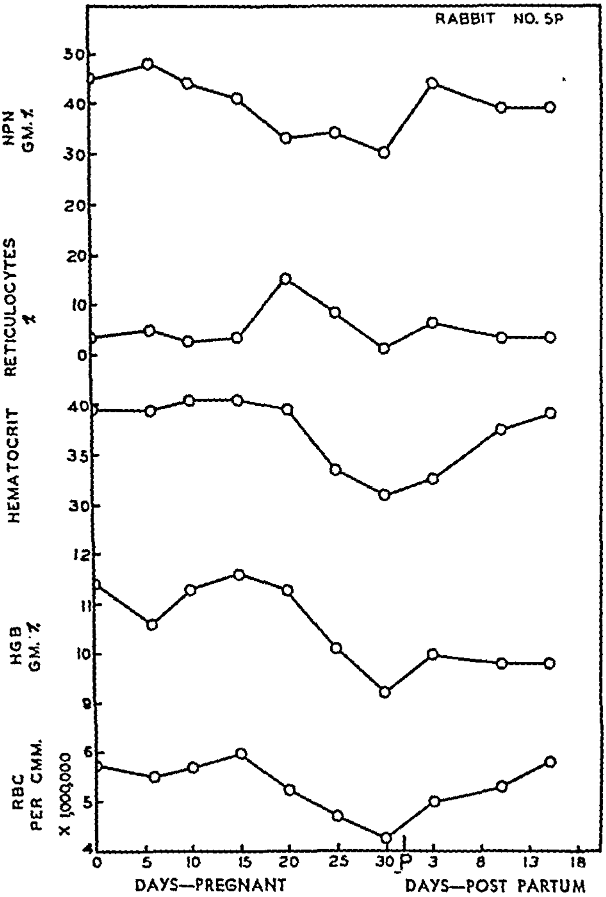

FIG. 1. Changes in the number of circulating erythrocytes, reticulocytes, hematocrit, hemoglobin concentration and non-protein nitrogen of the blood of Rabbit No. 5P during pregnancy and following parturition.

Analysis of the type of anemia present from data on several rabbits indicate that it was normochromic with a slight macrocytosis present. The mean corpuscular volume and mean corpuscular hemoglobin concentration showed no marked changes. A slight microcytosis occurred during recovery in the postpartum period.

TABLE 2. CHANGES IN THE SERUM NON-PROTEIN NITROGEN AND THE SERUM AND URINARY CHLORIDES DURING PREGNANCY IN THE RABBIT[1]

	Serum, non-protein nitrogen, m.%	Chlorides	
		Serum, milli-equivalents	Urine, gm. per 24 hours
Days pregnant			
0	42.6 ± 1.7[2]	105 ± 1.1	0.867 ± 0.177
5	44.5 ± 1.7	104 ± 1.3	—
10	42.4 ± 1.5	104 ± 1.1	0.750 ± 0.245
15	40.2 ± 1.9	102 ± 1.8	—
20	33.4 ± 2.1	104 ± 1.2	0.620 ± 0.34
25	32.6 ± 1.6	104 ± 1.1	0.566 ± 0.332
30	30.4 ± 1.5	107 ± 0.91	0.472 ± 0.307
Days post partum			
5	37.0 ± 1.4	105 ± 1.2	0.831 ± 0.284
15	40.0 ± 1.1	105 ± 0.98	0.906 ± 0.198

[1] Eight rabbits were used and a total of 10 pregnancies studied.

[2] Standard error $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

Water Balance During Pregnancy

The water intake and urine output was measured in a total of 18 pregnancies involving 15 rabbits. Throughout this study the animals were kept in metabolism cages and received food and water *ad libitum*. No lettuce

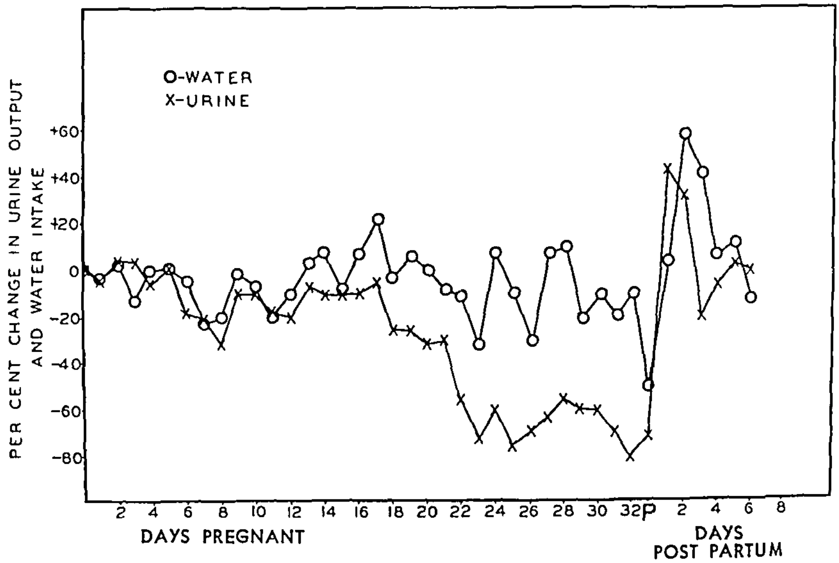

FIG. 2. Comparison of the urine output and water intake during pregnancy in rabbit No. 28. Both volume of urine and water are plotted as a per cent increase or decrease from the pre-mated levels. The decrease in urine output preceded the slight change in water intake and was much more marked.

was permitted the animals during the course of these experiments. All of the animals went into a positive water balance during the 2nd half of pregnancy but the degree of antidiuresis varied with the individual.

Figure 2 shows the data obtained on water balance studies during pregnancy in rabbit No. 28. Both the water intake and the urine output are plotted as a percentage change from the average values obtained prior to mating. Thus it is possible to plot the data on the same ordinate and to make a direct comparison of water balance. It is obvious that while the urine dropped markedly, the per cent decrease in water intake was not as great. Actually rabbit No. 28 showed only a small drop in water intake. In other rabbits a more marked decrease in water intake was obtained but in all instances the urine retention appeared first and was much more marked, indicating a definite retention of water.

Blood Constituents Following Treatment with Ovarian Extract

Data on the hemoglobin, hematocrit and cellular elements in the blood of the rabbit during treatment with relaxin may be seen in Table 3. A total of 8 rabbits were injected with 250 G.P.U. of the ovarian extract (assayed for its relaxin activity) three times daily for 12 days. The rabbits showed a normal erythrocyte count of 5,840,000 cells per cu. mm. Within six days after the start of treatment the count dropped to 5,310,000 and continued to drop so that by the 12th day the count had fallen to 4,546,000,

TABLE 3. CHANGES IN THE CELLULAR COMPONENTS AND HEMOGLOBIN OF THE RABBIT AFTER TREATMENT WITH 250 G.P.U. OF RELAXIN, 3 TIMES DAILY FOR 12 DAYS

	Erythrocytes, million per cu. mm.	Hemoglobin, gm. %	Hematocrit, %	Reticulocytes, %	Color index[1]
Length of treatment days					
0	5.84 ± 0.25[2]	12.4 ± 0.28	40 ± 0.77	2.8 ± 0.43	1.00
3	5.50 ± 0.14	11.1 ± 0.25	38 ± 1.5	3.3 ± 0.53	0.94
6	5.31 ± 0.21	10.5 ± 0.43	35 ± 1.8	4.5 ± 0.96	0.93
9	5.00 ± 0.27	9.4 ± 0.34	32 ± 1.0	5.6 ± 1.06	0.89
12	4.56 ± 0.28	9.1 ± 0.25	30 ± 1.1	5.9 ± 1.03	0.94
Days after cessation of treatment					
3	5.04 ± 0.42	9.8 ± 0.37	32 ± 1.4	7.3 ± 0.62	0.92
6	5.18 ± 0.34	10.3 ± 0.36	35 ± 1.6	7.6 ± 0.52	0.94
9	—	10.8 ± 0.38	36 ± 1.4	5.5 ± 1.77	—
24–28	5.80 ± 0.29	11.5 ± 0.24	39 ± 0.61	2.7 ± 0.70	0.93

[1] $$\frac{\text{gm. Hb./100 ml. (treated rabbit blood)}}{\text{gm./Hb./100 ml. (normal rabbit blood)}} \div \frac{\text{RBC count (treated rabbit)}}{\text{RBC count (normal rabbit)}}$$

[2] Standard error $= \sqrt{\dfrac{\Sigma d^2}{N(N-1)}}$

a decrease of 20.9%. A rise in the circulating red blood cells occurred within three days after the cessation of treatment with a return to the normal count by four weeks. A 25% drop in the hematocrit and 26.6% drop in the hemoglobin occurred concomitantly with the decrease in the circulating erythrocytes. The decline was progressive throughout the course of the treatment; the hemoglobin dropped from a normal of 12.4 gm % to 9.1 gm % and the hematocrit from 40 to 30. In both instances a rise was observed by the 3rd day after the cessation of treatment and the levels were back to normal 24 days after treatment had stopped.

The color index showed a slight but insignificant drop during treatment with the ovarian extract. In general, the color index indicated the parallel nature of the drop in hemoglobin concentration and circulating erythrocytes. The reticulocytes increased progressively throughout the course of treatment from a normal of 2.8% to 5.9%. During the first 6 days after cessation of treatment the reticulocytes continued to increase reaching an average concentration of 7.6%. Thereafter the number of cells declined to a normal level by the 24th day.

Table 4 contains the data on the changes observed in the non-protein nitrogen of the blood and in the serum and urinary chlorides. The most significant changes were observed in the concentration of the non-protein nitrogen which dropped from a pretreatment level of 40 mg. per cent to 33 mg. per cent by the 6th day of treatment with the ovarian extract and to 29 mg.% by the 12th day. Within three days after the cessation of treatment the nonprotein nitrogen rose to 36 mg. per cent and by 9 days had returned to normal. No particularly significant changes were noted in the serum chlorides. Normal rabbits in this experiment had an average

TABLE 4. CHANGES IN THE NON-PROTEIN NITROGEN OF THE BLOOD AND IN THE BLOOD AND URINARY CHLORIDES AFTER TREATMENT WITH 240 G.P.U. OF RELAXIN, 3 TIMES DAILY FOR 12 DAYS

	Non-protein nitrogen, mg. %	Chlorides	
		Serum, milli-equivalents	Urine, gm. per 24 hours
Treatment, days			
0	40 ±2.8	103 ±2.2	0.689 ±0.079
3	34 ±2.9	101 ±3.0	0.859 ±0.210
6	33 ±3.0	104 ±2.1	0.390 ±0.014
9	31 ±2.1	106 ±0.9	0.417 ±0.068
12	29 ±1.8	107 ±1.2	0.519 ±0.128
Days after treatment			
3	36 ±1.7	104 ±2.2	1.172 ±0.338
6	38 ±2.2	105 ±1.4	0.840 ±0.221
9	41 ±1.6	104 ±2.2	—
28	42 ±1.7	101 ±2.3	0.669 ±0.073

serum chloride of 103 ± 2.2 m. eq. which rose to 107 ± 1.2 m. eq. This slight increase is not significant though further studies are needed before an effect on serum chlorides can be ruled out. Urinary chlorides were determined on a limited number of rabbits and the results were highly variable. The normal rabbits showed a urinary chloride excretion of 0.68 ± 0.079 gm. per 24 hours. This fell to 0.390 ± 0.014 gm. per 24-hours after 6 days of treatment with relaxin and then rose to 0.519 ± 0.128 gm.

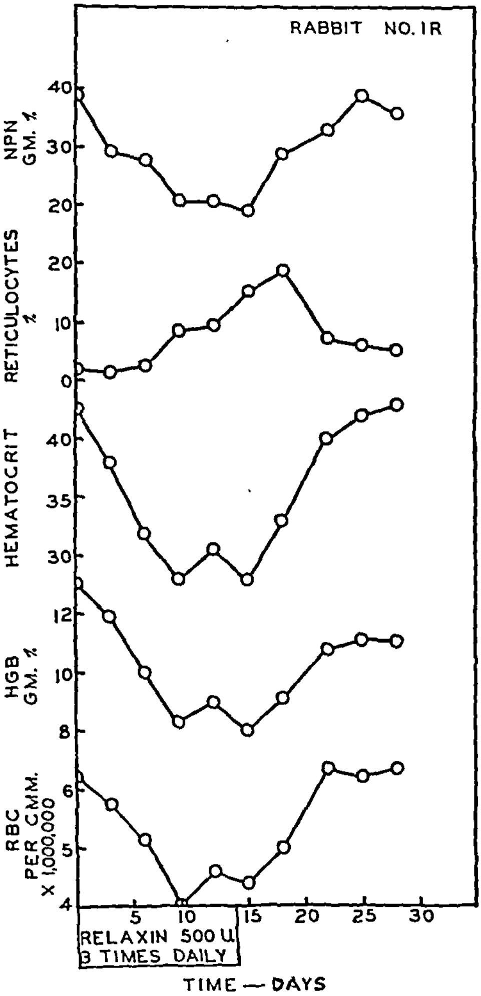

FIG. 3. The effect of a water soluble ovarian extract (relaxin) on the non-protein nitrogen, reticulocytes, hematocrit, hemoglobin, and erythrocytes of the blood of a normal rabbit.

per 24-hours on the 12th day of treatment. On the third day after treatment the excretion rose to 1.172 ± 0.338 gms. per 24-hours and then returned to normal by the 24th day.

Rabbit No. 1R was injected with 500 relaxin G.P.U. of ovarian extract three times daily for 14 days (Fig. 3) and showed the same pattern as noted for the lower dose except for a more acute response. This would tend to indicate that some type of a dose-response relationship exists. A sharp, and parallel decline in the circulating erythrocytes, hemoglobin and hematocrit may be observed. The maximum response occurred on the 10th day, although treatment was continued for 4 more days. Immediately upon the cessation of treatment all values began to return to normal. The nonprotein nitrogen showed a marked drop of 48%, i.e. from 39 gm. % to 20. The reticulocytes showed a marked increase on the 10th day of treatment and continued to rise in the post treatmént period reaching a peak of 18% on the 4th day after treatment was stopped.

It may be noted that no marked changes occurred in either the mean, corpuscular hemoglobin or mean, corpusculr hemoglobin concentration. A slight drop in the mean corpuscular volume occurred but this was only of the order of 5% and its significance is highly questionable. It would appear that the anemia produced by the ovarian extract is both normochromic and normocytic.

Water Balance During Treatment With the Ovarian Extract

Figure 4 shows the results obtained on the water intake and urine output following treatment with 500 G.P.U. relaxin (ovarian extract) for 12 days. The data are expressed as a per cent change from the pretreatment levels and are plotted on the same ordinates. It is obvious from the results that, while both the water intake and urine output drop, the decrease is much more marked with regard to the urine. Furthermore, the decrease in urine output occurred several days prior to the decrease in water intake. Hence the initial effect of the extract on the water balance would appear to be a water retention.

DISCUSSION

It is apparent from these results that the rabbit in common with several other species develops an anemia during pregnancy. The erythrocytes decreased from an average of 5.95 ± 0.083 million per cu. mm. to 4.71 ± 0.12 million per cu. mm. This is a 20% drop in the circulating erythrocytes which is paralleled by a concomitant drop in hemoglobin (16.7%) and hematocrit (17%). The anemia is normochromic with a slight tendency toward macrocytosis. The parallel nature of the decrease in erythrocytes

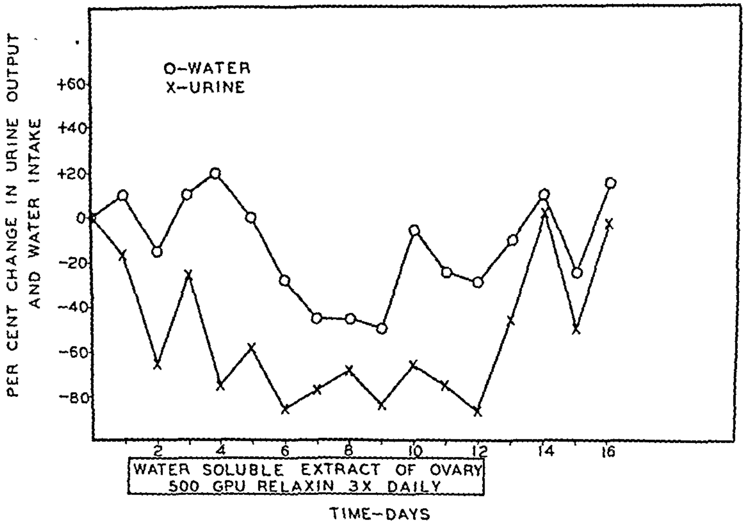

FIG. 4. Changes in water intake and urine output in Rabbit No. 94 after treatment with a water soluble extract of the ovary of the pregnant sow. The data are plotted as a per cent change from the pretreatment levels and indicate a decrease in both water intake and urine output. However the decrease in urine is more marked and occurs first indicating a water retention.

and hemoglobin is also indicated by a lack of change in color index during pregnancy. The reticulocytes increased from a normal of 2.1% to 6.8% on the 20th day of pregnancy and then fell in spite of the increasing severity of the anemia. A second peak in reticulocytes was seen during the recovering phase i.e. on the 5th day after delivery. No significant changes were noted in the white blood cell count or differential.

Treatment with the ovarian extract (250 G.P.U.) for 6 and 12 days induced changes of the same order of magnitude as seen during pregnancy. The 6-day treatment caused a 25.8% drop in the nonprotein nitrogen, 16.9% drop in the erythrocytes and a 21.9% drop in the hematocrit. Treatment for 12 days with the same dose caused a drop of 29.6% in the erythrocytes, 23.1 in the hemoglobin and 24.4 in the hematocrit. Doubling the dose of the ovarian extract increased the severity of the anemia, resulting in a 36.7% drop in the erythrocytes, 36.1% in hemoglobin and 33.9% in hematocrit.

It is of interest to note that the ovarian extract was able to produce a drop in the nonprotein nitrogen of the blood. The decrease was approximately of the same order of magnitude as seen during pregnancy i.e. a 29.5% drop during pregnancy and a 25.8 to 36.7% drop following treatment with the ovarian extract. While disturbances in protein metabolism during pregnancy have been observed by others (Lindeboom, 1948;

Hamilton and Higgins, 1949), it is intriguing to note that the ovarian extract was not only able to produce an anemia but also produce a drop in the nonprotein nitrogen of the blood.

The present results indicate that during pregnancy there is a disturbed water balance. In spite of the decreased water intake in some rabbits, all of them showed some degree of water retention during the final trimester of gestation. Such a condition has been described for women (Chesley, 1944) with an indication of changes in the ratio of extravascular fluid volume and plasma volume (Caton, Roby, Reid and Gibson, 1949). Recently van Dyke (1950) has questioned the antidiuretic action of the ovarian extract used in this study (Zarrow, 1949) on the grounds that the antidiuretic response was the result of a reduced water intake. While a reduced water intake is apparent during pregnancy and also during treatment with the ovarian extract, nevertheless the reduction in urine is far greater, resulting in a marked water retention. Furthermore the decrease in urine output consistently preceded the drop in water intake. This is apparent in Figures 2 and 4 which show the per cent change in the urine and water. The ability of the ovarian extract to produce an anemia, a drop in nonprotein nitrogen, and a positive water balance plus the similarity in the pregnancy-anemia and the extract-induced-anemia would tend to identify this ovarian extract as the possible etiologic factor.

Though relaxin preparations were used in this investigation as the ovarian extract it is apparent that the anemia-inducing effect is not due to relaxin. A number of highly purified preparations of relaxin failed to show any water retention or anemia-inducing action. Recently Frieden and Hisaw (1950) suggested that relaxin may exist as a complex molecule which retains different physiological activities after splitting. Further work is needed along these lines especially in the purification of the different water soluble factors.

SUMMARY AND CONCLUSIONS

Studies were made on the erythrocyte count, leucocyte count, differential count, reticulocytes, hemoglobin concentration, non-protein nitrogen, blood and urine chlorides and water balance of the grey chinchilla rabbit throughout pregnancy and after treatment with water soluble extracts of the ovary. During pregnancy the rabbits developed a normochromic, slightly macrocytic type of anemia, with a 20% drop in erythrocytes a 16.7% drop in hemoglobin and a 17% drop in hematocrit. The reticulocytes showed a sharp peak on the 20th day of pregnancy in spite of the increasing severity of the anemia. With the onset of the anemia, the rabbits developed a definite water retention. The non-protein nitrogen of the blood fell during pregnancy and recovered shortly after parturition. The chloride secretion in the urine dropped though no marked changes were noted in the blood chlorides. It may be concluded that the rabbit develops an anemia of pregnancy concomitant with a water retention.

Treatment with the ovarian extract produced a definite retention of water and an anemia similar to that noted during pregnancy. The minimal dose used in these studies produced a drop of 21% in the circulating erythrocytes, a 23% drop in the hemoglobin, a 24% drop in the hemocrit, and a 30% drop in the nonprotein nitrogen of the blood. The animals showed a marked reticulocytosis and a tendency to chloride retention. Treatment with twice the above amount of ovarian extract, produced a more severe anemia and a greater drop in the nonprotein nitrogen of the blood. Both doses induced a positive water balance with a definite retention of water in spite of a slight decrease in water intake. No marked changes appeared in the serum chlorides. It is suggested that the anemia may be caused by a hemodilution due to an action of the water soluble extract of the ovary and that the anemia-inducing action of the ovarian extract is not due to relaxin.

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PITUICYTES AND THE ORIGIN OF THE ANTIDIURETIC HORMONE[1]

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INTRODUCTION

THE question of the secretory activity of the tissue elements in the pars nervosa and more specifically of the pituicytes has been treated by most authors (Pickford, 1945; O'Connor, 1947; Harris, 1948; Collin and Stutinsky, 1949) as an open one, since the cells of the neurohypophysis do not possess cytological characteristics peculiar to gland cells, and since past attempts to link the production of posterior lobe hormones to microscopically visible changes in the pituicytes had failed. Gersh (1938, 1939) and Gersh and Brooks (1941), for instance, believed that they had shown a relationship between the state of dehydration of their experimental animals and the presence in the pituicytes of granules which they could stain with osmic acid. However, Hickey, Hare and Hare (1941), De Robertis and Primavesi (1942), and Desclin (1947) were unable to confirm these findings. The observations of Wang (1938) which have been frequently cited as supporting the concept of a secretory function of the pituicytes were based on sections stained with the Hortega method which is not suitable for the study of the cytology of secretion.

A new approach to this problem was suggested by the work of Bargmann (1949 a,b), Bargmann and Hild (1949), Bargmann, Hild, Ortmann, and Schiebler (1950), Ortmann (1950, 1951), Hild (1951 a,b; 1952), Kratzsch (1951), Hild and Zetler (1951 a,b; 1952 a,b; 1953 a,b) who demonstrated by histological and experimental methods the presence in the neurohypophysis of material which can be selectively stained with chrome-alum hematoxylin and which is invariably associated with the vasopressor, antidiuretic, and oxytocic hormones. If, as Ranson and his coworkers believed (Fisher, Ingram and Ranson, 1938; Ranson, Fisher and Ingram, 1938; Magoun, Fisher and Ranson, 1939; Ranson and Magoun, 1939), the pituicytes are concerned with the elaboration of the posterior lobe hormones, these elements should be implicated in the production of the stainable material. This question has been answered by Ortmann (1950,

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1951), Hild (1951 a,b; 1952) and Kratzsch (1951) who found no evidence to indicate that the pituicytes produce the stainable substance with which the posterior lobe hormones are associated.

However, Pickford (1952) as well as the investigators who participated in a meeting of the Society for Endocrinology devoted to the "Posterior Pituitary Gland" (Lancet, July 5, 1952, pp. 22–23) do not refer to the evidence offered by Ortmann, Hild and Kratzsch, but continue to base their conclusions on the hypothesis of Ranson.

Although we feel that the data presented by Ortmann, Hild and Kratzsch are valid, we restudied the problem in rats which had been subjected to dehydration both by withholding of drinking water, and by administration of a salt load. Under such conditions the demand for the antidiuretic hormone is greatly increased, since the animals rapidly use it up in an effort to conserve water (Gilman and Goodman, 1937). The pituicytes, if they have a secretory function, should consequently be at the height of their activity in animals whose store of antidiuretic hormone has been depleted by dehydration. In addition, the pituicytes should be more clearly visible in the neurohypophyses of dehydrated animals since the pars nervosa of animals so treated shows a marked depletion of the stainable material. Any sign of secretory activity on the part of the cells should consequently be easily discernible.

MATERIAL AND METHODS

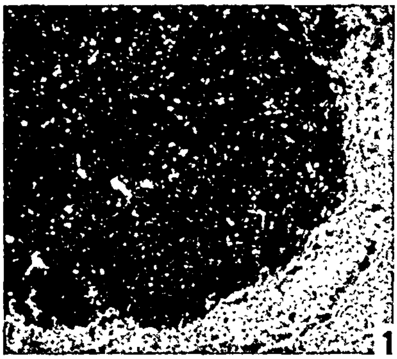
White rats of the Sprague-Dawley and University of Denver Strains were dehydrated in one of two ways: Some received no drinking water for periods up to 13 days, others were given a 2.5% solution of sodium chloride instead of drinking water for 13 days. The period of dehydration by administration of salt had been previously determined in several animals as one resulting in maximum depletion of stainable material in the posterior pituitary. Following the period of dehydration, the animals were given free access to tap water and then sacrificed at regular intervals.

It is important, as Rothballer (1952) has shown, that the rats be killed without allowing them to become unduly excited. Unspecific stress such as that brought about by prolonged handling or by the prick of a hypodermic needle in the administration of an anesthetic leads to the release of antidiuretic hormone within a few minutes. Therefore, the rats were taken from their cages and immediately transferred to a jar containing chloroform.

Fixation of the tissues was accomplished by perfusion through the heart with Zenker-Formol. The pituitaries were embedded in paraffin, sectioned at 5 micra, and stained with Gomori's chrome-alum hematoxylin phloxin (Gomori, 1941; Bargmann, 1949 a,b).

EXPERIMENTS AND OBSERVATIONS

The need for water conservation by the "intact" organism, which occurs in water deprivation, is a stimulus for the release of the antidiuretic hormone (Gilman and Goodman, 1937). In dehydrated animals, the amount of antidiuretic hormone in the posterior lobe is consequently reduced (Hild and Zetler, 1952 b). Histologically, this reduction of hormone content expresses itself in the disappearance of a substance which stains selectively blue with Gomori's chrome hematoxylin-phloxin (Figs. 1 and 2) and to which the antidiuretic hormone is closely bound (Ortmann, 1951; Hild and Zetler, 1952 b). The extent to which hormone content and stainable material are reduced is directly related to the severity of the dehydration. Animals with maximal reduction of hormone content of the posterior lobe show a nearly complete depletion of the stainable substance. When, after reaching this stage, animals are again given drinking water, the stainable material reappears in the posterior pituitary (Figs. 3 to 5). If the pituicytes are concerned with the elaboration of this material, it should first become visible in these cells.



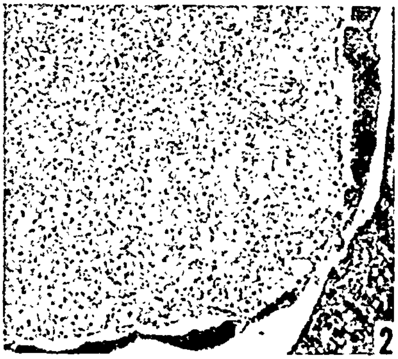


FIG. 1. Pituitary of a normal rat. The pars nervosa is filled with material staining deep blue. Zenker-formol, paraffin, 5μ, Gomori's chrome hematoxylin phloxin method, photomicrograph. ×60.

FIG. 2. Pituitary of a rat dehydrated by the ingestion of 2.5% sodium chloride solution for 13 days. The pars nervosa is depleted of stainable material. Technique and magnification as in Figure 1.

1. Animals deprived of drinking water

a. Six female Sprague-Dawley rats, 4 months old litter mates, were

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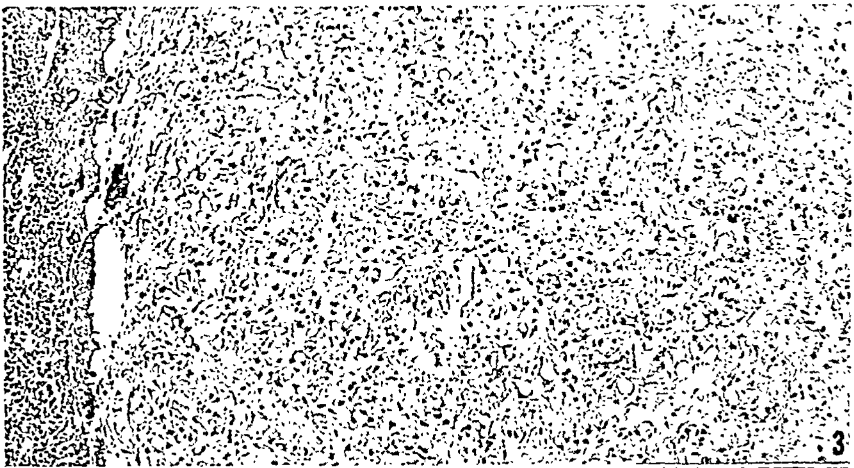
FIG. 3. Pars nervosa of a rat depleted of stainable material after 13 days of ingestion of a 2.5% solution of sodium chloride. Vascular perfusion with Zenker-formol, paraffin, 5μ, Gomori's chrome hematoxyline phloxine, photomicrograph. ×100.

FIG. 4. Re-accumulation of stainable material in the pars nervosa of a rat which was allowed to drink tap water for 2 days following 13 days of dehydration (2.5% NaCl). Technique and magnification as in Figure 3.

FIG. 5. Further re-accumulation of stainable material in the pars nervosa of a rat which was allowed to drink tap water for 4 days following 13 days of dehydration (2.5% NaCl). Technique and magnification as in Figure 3.



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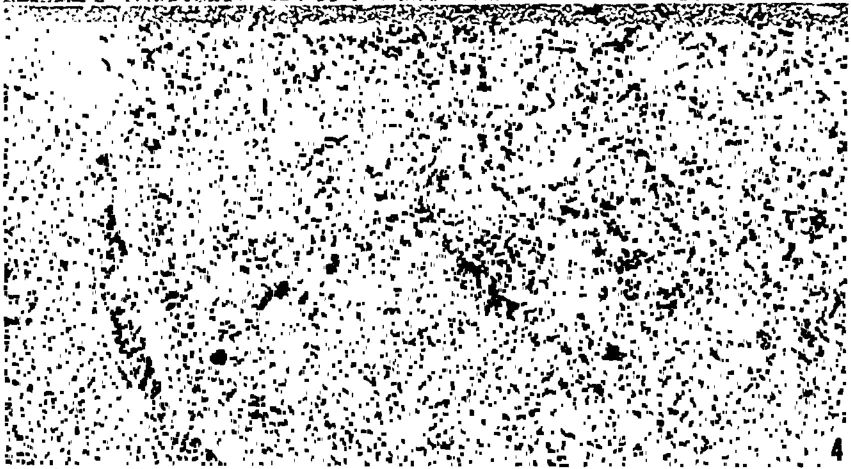

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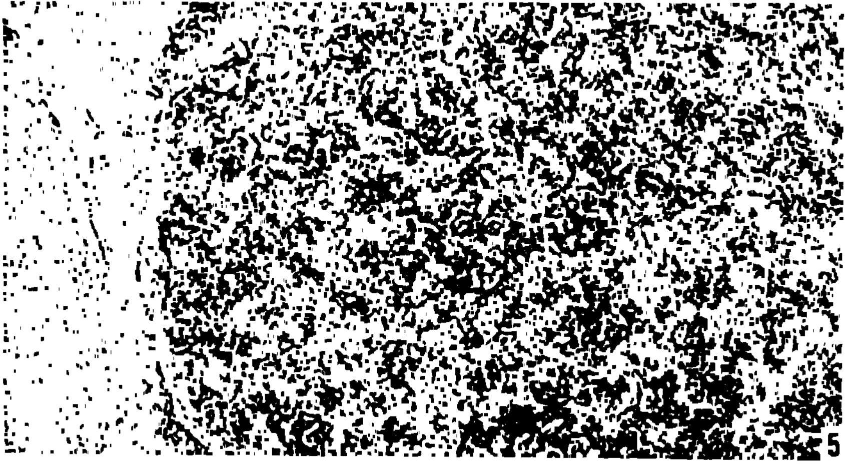

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kept without drinking water for 6 days in July. One animal was sacrificed on the sixth day, the others were given free access to water and were killed 2, 3, 4, 5, and 7 days after the termination of the thirst period.

b. Six female Sprague-Dawley rats of about the same age, but not litter mates, were deprived of water for 13 days in August. Two animals died on the 13th day, a third was killed. The survivors were given water and were then fixed by perfusion with Zenker-formol 1 and 2 days after they had again received drinking water. The sixth rat did not recover and died.

c. Nine female rats of the University of Denver strain which were of about the same age but not litter mates, were deprived of drinking water for 9 days in September. Only three animals survived, of which 2 were killed one and two days after having been given water to drink.

As previously reported (Scharrer, 1952), in none of these animals did the pituicytes in sections stained with the Gomori technique show the faintest granule. The stainable substance did reappear in animals who received drinking water after a period of thirst, but there was no sign that the pituicytes produced it.

We have observed, however, that dehydration produced by the withholding of drinking water is not as suitable a method of depleting the posterior pituitary of its antidiuretic hormone and stainable substance as that produced by the administration of hypertonic saline. During prolonged thirst, the animals refuse to eat and their eyes, nose, and paws become hemorrhagic. There is also much variation in the tolerance of individual animals to the experimental procedure depending upon the moisture content of the air, the climate, and the time of the year at which the animals are subjected to dehydration. Thus, the degree of depletion of the pars nervosa cannot be gaged as accurately in thirsted animals as in those which are given salt solutions of known concentrations. In the latter method of depletion the rats continue to eat, although they may do so to a lesser degree, and they show fewer signs of acute distress during an experimental period of 13 days. Except for very isolated cases, the depletion of the posterior pituitary can be accomplished without the loss of any experimental animals. Consequently, the following sets of experiments were carried out.

2. Animals given a 2.5% solution of sodium chloride instead of drinking water

a. Five young adult rats, litter mates, of the Sprague-Dawley strain were used in order to substantiate further earlier findings which indicated that after 13 days of administration of a 2.5% solution of sodium chloride the depletion of the substance stainable with chrome-alum hematoxylin is usually complete. A study of microscopic sections of the pituitaries of these animals revealed no sign of activity on the part of the pituicytes.[2]

b. Six adult rats, litter mates, of the Sprague-Dawley strain were subjected to saline dehydration for a period of 13 days after which time they were again allowed tap water. One rat was sacrificed on the first day, 2 rats on the second day, 2 on the third day, and 1 on the fourth day following reinstitution of tap water in the diet.

c. Eight adult rats, litter mates, of the Sprague-Dawley strain were subjected to the same experimental procedure as described in 2b. Two rats were perfused with Zenker-formol on the fourth day, 2 on the fifth day, 2 on the sixth day, and 2 on the ninth day following the period of dehydration.

Upon substitution of water for the hypertonic saline a progressive reaccumulation of secretory material was noted in the pars nervosa (Figs. 3 to 5). The first few days following the period of dehydration might be considered crucial with respect to the activity of the pituicytes. At no time were intracellular granules stainable with Gomori's chrome hematoxylin seen in any of these cells (Fig. 6). Rather, the stainable material

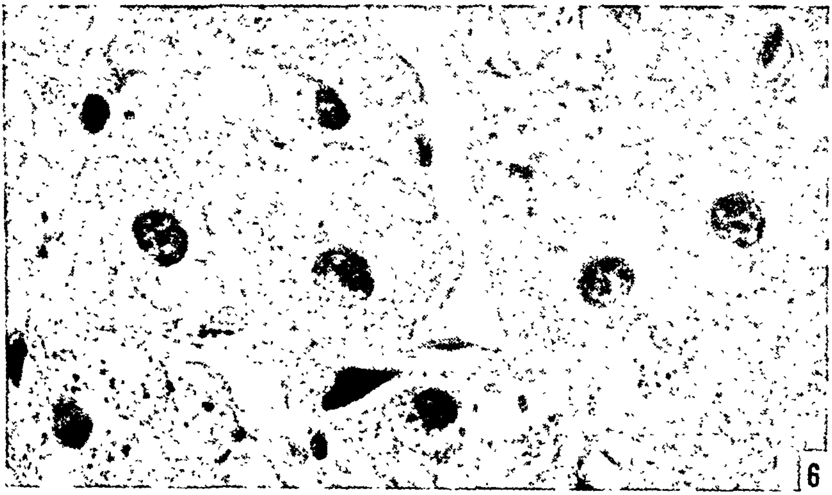

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FIG. 6. Cells of the pars nervosa of a rat which had been kept without drinking water for 9 days. There is no indication that these cells elaborate any material stainable with chrome hematoxylin. Vascular perfusion with Zenker-formol, paraffin, 5μ, Gomori's chrome hematoxylin phloxin. ×1100.

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[2] Chambers (1945), Selye (1950), Ortmann (1951) and Hild (1951 a) have reported the occurrence of numerous mitoses in the neural lobes of dehydrated rats and frogs. The significance of these mitoses is not clear. They do not occur in water starved dogs (Personal communication of Dr. W. Hild).

can first be seen to accumulate in the perivascular areas which Vazquez-Lopez (1942) described as clear spaces and in which the fibers of the hypothalamo-hypophysial tract terminate.

DISCUSSION

The explanation of the mechanisms operating in the clinical entity known as diabetes insipidus proposed by Ranson and his coworkers is based in part on the presumed innervation of pituicytes by nerve fibers derived from hypothalamic nuclei, and in part on the premise that the pituicytes elaborate the pharmaco-dynamic factors obtainable from the neurohypophysis. This interpretation is still held by many workers in the field (Holzer and Hölscher, 1949; Noble, Plunkett and Taylor, 1950; Harris, 1951; Pickford, 1952), although there is ample evidence to the effect that the hormones of the posterior lobe are the products of neurosecretory cells in the hypothalamus. We feel, therefore, that a clarification of the role of the tissue elements in the elaboration of the hormones attributed to this part of the pituitary is of importance.

Ranson and his coworkers believed that the cells producing the antidiuretic hormone could no longer function when deprived of nervous impulses originating in the nucleus supraopticus and probably also in the nucleus paraventricularis. However, Gagel and Mahoney (1936), Mahoney and Sheehan (1936), and Keller (1942) showed that diabetes insipidus does not necessarily result from interruption of the tractus supraoptico-hypophyseus. According to these authors, only lesions in the immediate vicinity of the supraoptic nucleus, cause permanent diuresis; section of the pituitary stalk halfway between hypothalamus and pituitary are not followed by more than a temporary disturbance of the excretion of water. Another aspect concerns the observation of Rasmussen (1938) who pointed out that there are many more fibers in the tractus supraoptico-hypophyseus than would be necessary to innervate the cells of the neurohypophysis. Furthermore, only some of the investigators were able to demonstrate actual contact between nerve fibers and pituicytes (Brooks and Gersh, 1938; Roussy and Mosinger, 1940; Tavares de Sousa, 1943; Romieu and Stahl, 1950). Others state that the pituicytes do not receive nerve terminals from the tractus supraoptico-hypophyseus (Hair, 1938; Rasmussen, 1938; Romeis, 1940; Vazquez-Lopez, 1942; Green 1948). Actually, Vazquez-Lopez (1942), Hagen (1950) and Stotler (1952) have shown that many fibers end at the blood vessels of the posterior pituitary. These fibers do not innervate the vessels, but store neurosecretory material which contains the posterior lobe hormones (Bodian, 1951; Hild, 1952). Consequently, there is little if any basis for the assumption that the pituicytes are stimulated by nervous impulses originating in the nucleus supraopticus or paraventricularis and that such stimulation causes the pituicytes to produce the posterior lobe hormones.

As to the second premise of Ranson, little attention had been paid in the past to the pars nervosa as a possible site of secretory activity, especially since Herring (1908) had presumably established that the hormones found in the posterior pituitary are secreted by cells of the pars intermedia which migrate into the pars nervosa. Cushing (1933, p. 541) emphatically stated, that "it is scarcely conceivable that the neural core of the lobe is capable independently of elaborating a hormone" and subscribed to the theory that the active principles found in the posterior pituitary are derived from the investing pars intermedia. On the other hand, Biggart (1935, 1936) preferred the cells of the pars tuberalis as the site of hormone production. Recently, Büchner (1950) revived the views of Herring and Cushing and combined them with the theory of Ranson, by suggesting that the basophils of the pars intermedia migrate into the neurohypophysis and become subject to the nervous control of the nucleus supraopticus via the tractus supraoptico-hypophyseus. Viewed in this way the function of the posterior lobe of the pituitary becomes indeed "one of the most difficult problems of the biology of the endocrine system" (Büchner, p. 414). It was not until Bucy (1930, 1932) singled out the glia cells in the ox pituitary by giving them a special name ("pituicytes") that the possibility of a secretory activity of the tissue elements of the pars nervosa was given serious consideration. According to Vazquez-Lopez (1942) and Collin and Stutinsky (1949) the term "pituicyte" is, however, not justified. It is only in the ox pituitary which Bucy happened to study, that these cells differ somewhat in their shape from astrocytes or oligodendroglia. In the horse Vazquez-Lopez found no features that would distinguish the glia cells of the neurohypophysis from those of other parts of the central nervous system. The same seems to hold true for the cat and the dog (Sanz Ibáñez, 1934). Spatz (1951) considers the "pituicytes" as incompletely differentiated glia cells. On the other hand, Shanklin (1944) states the following with respect to the pituitary of the pig: "There is a marked similarity between the developing stages of the pituicytes and astrocytes. Each originates from supportive spongioblasts and may pass through bipolar, unipolar and multipolar stages. Like astrocytes they have vascular processes and foot plates. Pituicytes differ from astrocytes, oligodendroglia and microglia for they have many connections between one another whereas the others do not." In the human neurohypophysis various types of pituicytes have been described (Romeis, 1940).

However, the question of the shape of these cells has little if any relationship to the problem under discussion here. Even if a type of glia cell of the kind described by Bucy were to occur generally in the mammalian neurohypophysis, it has never been made clear why a secretory function should be attributed to these cells. The possibility that glia elements in general are potential sources of hormones cannot be excluded (Achúcarro, 1913; Griffith, 1940), but at present no experimental evidence is available in support of such a hypothesis. In the particular case of the cells of the neurohypophysis, our findings in addition to those of Hild, Ortmann and Kratzsch would seem to constitute convincing evidence to show that these cells do not produce the hormones obtainable from the posterior lobe.

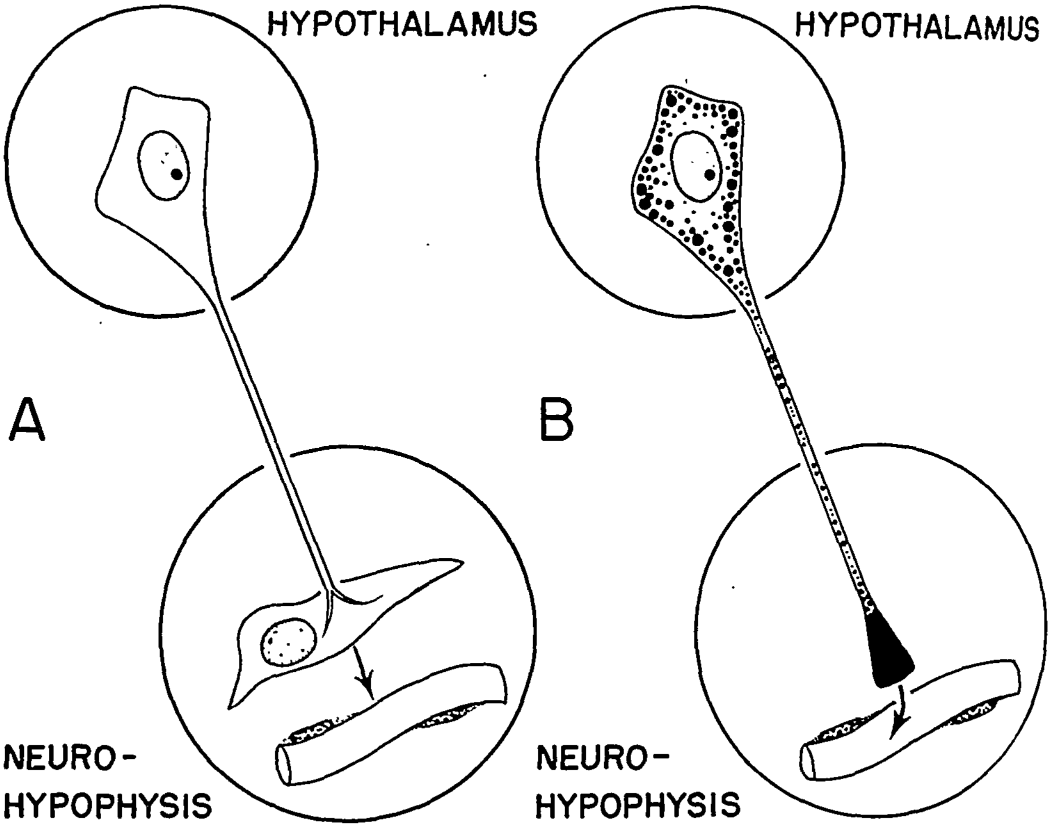

Fig. 7. Diagrammatic representation of two concepts concerning the site of origin and the mode of release of the posterior lobe hormones. A. The currently held view, based mainly on the investigations of Ranson and his coworkers, assumes that cells in the neurohypophysis elaborate and release the posterior lobe hormones under nervous control exerted by hypothalamic nuclei. B. Newer evidence indicates that the posterior lobe hormones are associated with the neurosecretory material produced by nerve cells in the hypothalamus; this material passes along the nerve fibers into the neurohypophysis where it is stored until released into the circulation.

Since, then, it can no longer be maintained that the supraoptic and paraventricular nuclei innervate hormone-producing cells in the neurohypophysis, the theory of Ranson should be replaced by the concept of the neurosecretory origin of the posterior lobe hormones (Fig. 7). According to this concept the nerve cells of the supraoptic and paraventricular nuclei produce the posterior lobe hormones which pass along the fibers of the tractus supraoptico-hypophyseus to attain the neurohypophysis. They are stored there in the nerve terminals[3] from which they are released into the blood vessels.

SUMMARY

The role of the pars nervosa of the pituitary, and more specifically of the pituicytes in the secretion of the antidiuretic hormone, was reexamined.

The antidiuretic hormone occurs in the supraoptic and paraventricular nuclei of the hypothalamus, in the fibers of the tractus supraopticohypophyseus, and in the neurohypophysis in association with material which stains selectively with Gomori's chrome hematoxylin phloxin method (Hild and Zetler, 1951 a,b; 1952 a,b; 1953 a,b). If the pituicytes play a role in the elaboration of the antidiuretic hormone, they should be active in secreting this stainable material in animals which had been dehydrated both by the withholding of drinking water and by the administration of sodium chloride of known concentrations. It was found, however, that the pituicytes have no part in the elaboration of the stainable material and consequently of the antidiuretic hormone.

A critical analysis of the evidence on which Ranson's theory of the function of the posterior pituitary was based, leads one to the conclusion that this theory should be abandoned in favor of the concept of the neurosecretory origin of the antidiuretic hormone.

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[3] The possibility that the posterior lobe hormones are produced by the nerve endings in the neurohypophysis was first suggested by Rasmussen (1938), later discussed by O'Connor (1947) and recently again proposed by Bodian (1951). However, all the available evidence indicates that the hormones are merely stored, but not elaborated in the nerve terminals.

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THE *IN VIVO* METABOLISM OF CORTISONE[1]

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THE application of paper chromatography and infra-red spectrometry has permitted the isolation and identification of the major C-21 and C-19 metabolites excreted in urine, following the administration of adrenosterone (Savard *et al.*, 1953) hydrocortisone and 21-desoxycortisone (Burstein *et al.*, 1953). It has been shown that the C-21 steroids are reduced in the human body to the pregnane (5β) and transformed to the corresponding C-19 etiocholane (5β) derivatives. On the other hand, the Δ^4-3 keto system in adrenosterone is converted predominantly into derivatives of the androstane (5α) series, which indicates that the ethyl side chain in position 17 of these steroids appears to direct the reduction of the $\Delta^{4,5}$ double bond to the 5β form *in vivo.*

The *in vivo* metabolism of cortisone has been described by Dobriner *et al.* (1950, 1951) and Lieberman *et al.* (1951). However, no details were given as to the relative quantities of the metabolites excreted.

In view of the importance of cortisone in endocrinological research and in therapeutics, the study of the metabolism of orally administered cortisone acetate to a normal man has been undertaken. The present paper demonstrates the conversion of cortisone: 1) to pregnane-3α,17α,21-triol-11,20-dione and pregnane-3α,11β,17α,21-tetrol-20-one; 2) to hydrocortisone, and 3) to 17-ketosteroids of the etiocholane (5β) configuration bearing an oxygen function at carbon 11.

METHODS

A 26 year old male patient who had been successfully treated for hepatitis 4 months previously was used in this study when his clinical picture was normal.[2] An oral dose of 500 mg. of cortisone acetate[3] was administered daily for 5 days.

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[2] We are indebted to Dr. G. W. Thorn for the clinical aspects of this study.

[3] The cortisone acetate was generously supplied to Dr. G. W. Thorn for this study by Merck & Company, Rahway, New Jersey.

Steroid isolations were performed on a urine sample which represented 1.8 gm. of the administered cortisone acetate. A 24-hour urine collection just prior to the cortisone treatment served as a control. The steroid conjugates were hydrolyzed, and the ketonic extracts chromatographed on paper in the manner previously described (Burstein *et al.*, 1953). The isolated steroids were identified by chromatographic mobility on paper, melting points, and infra-red analyses. We are indebted to Dr. Harris Rosenkrantz for the valuable interpretation of the infra-red spectra. The melting points were determined by the Kofler Micro Hot Stage melting point apparatus, and are recorded uncorrected.

EXPERIMENTAL AND RESULTS

Control Urine: The corticosteroid fraction (which stayed at the starting line following chromatography in the toluene-propylene glycol (T.-P.G.) system for 4 hours) of the ketonic material of the 24 hour control urine contained 1.2 mg. of formaldehydogenic steroid. The quantities of the 17-ketosteroids contained in the zones having the mobility of the order of 11β-hydroxyandrosterone (0.6 mg.) and etiocholane-3α-ol-11,17-dione (0.5 mg.) were insufficient for crystallization and infra-red spectrometry. No etiocholane-3α,11β-diol-17-one was detected. Etiocholane-3α-ol-17-one (5.2 mg.) and androsterone (3 mg.) were isolated in crude form and identified by their infra-red spectra.

Cortisone administration urine: The results obtained with the cortisone urine are listed in Table 1. In the ring A saturated corticosteroid fraction, which was rechromatographed in a chloroform-formamide system (Schindler and Reichstein, 1951), pregnane-3α,11β,17α,21-tetrol-20-one and pregnane-3α,17α;21-triol-11,20-dione were obtained. The latter steroid was crystallized directly but the former steroid was acetylated and rechromatographed in the ligroin-propylene glycol (L.-P.G.) system (52 hours).

TABLE 1. ISOLATION OF STEROIDS FROM CORTISONE URINE

Chrom. zone	System	Hrs.	Location of zone: dist. from starting line cm.	Weight of isolated steroid		Compound	Identifying characteristics
				Crude, mg.	Crystal, mg.		
1-I	Ch.-F.*	12	5.5– 8.5	4.6		Pregnane-3α,11β,17α,21-tetrol-20-one	Mobility§
1-I acet-	L.-P.G.†	52	3.2– 5.8	2.0	1.0	Pregnane-3α,11β,17α,21-tetrol-20-one-3α,21-diacetate	M.P. 208–215°; Mobility; Infra-red spectrum
1-II	Ch.-F.	12	12.5–17.2	44	18	Pregnane-3α,17α,21-triol-11,20-dione	M.P. 194–198°; Infra-red spectrum
1-III	T.-P.G.‡	72	5.0– 8.1	5		Hydrocortisone	Mobility; Infra-red spectrum
1-IV	T.-P.G.	72	9.0–12.0	1.1		Unknown	[illegible]
1-V	T.-P.G.	72	13.2–16.0	2.1		Cortisone	[illegible]
1-VI	T.-P.G.	72	23.0–27.0	0.7		Unknown	[illegible]
2-I	L.-P.G.	96	1.5– 3.0	11.3	4.3	Etiocholane-3α,11β-diol-17-one	M.P. 232–36°; Infra-red spectrum
2-II	L.-P.G.	72	6.0–12.0	9.0	3.2	Etiocholane-3α,ol-11,17-dione	M.P. 180–82°; Infra-red spectrum
3-I	L.-P.G.	24	12.5–18.0	6.3		Δ^9-Etiocholene-3α-ol-17-one	Mobility; Infra-red spectrum
3-II	L.-P.G.	24	23.7–29.0	4.4		Δ^9-Androstene-3α-ol-17-one	Mobility; Infra-red spectrum

* Chloroform-Formamide.
† Ligroin-Propylene-Glycol.
‡ Toluene-Propylene-Glycol.
§ Possessing the mobility of the order of the respective compound.

Both pregnane compounds were identified by their infra-red spectra. In the Δ^4-3-keto-corticosteroid fraction, hydrocortisone and cortisone were found and identified by their chromatographic mobility on paper and infra-red spectra. The hydrocortisone gave a strong greenish fluorescence in sulfuric acid and no fluorescence was obtained with the isolated cortisone. Two other blue tetrazolium reducing compounds were observed on the chromatogram. One was located between the hydrocortisone and cortisone zones (Zone 1-IV) and the second occurred immediately ahead of the cortisone zone (Zone 1-VI). The quantity of these substances was insufficient for further identification.

Among the 17-ketosteroids, etiocholane-$3\alpha,11\beta$-diol-17-one (from Zone 2-I), etiocholane-3α-ol-11,17-dione (from Zone 2-II), Δ^9-etiocholene-3α-ol-17-one (from Zone 3-I) and Δ^9-androsten-3α-ol-17-one (from Zone 3-II) were isolated and identified by their mobilities, melting points and infra-red spectra (Table 1). Neither etiocholane-3α-ol-17-one nor androsterone were found in the cortisone urine, although significant quantities of these steroids were excreted during the control period (Table 2).

TABLE 2. STEROID ISOLATION IN CORTISONE METABOLISM STUDY

Steroid	Quantity isolated (crude) mg.	
	Control[1] urine	Cortisone urine
Pregnane-$3\alpha,11\beta,17\alpha,20$-tetrol-20-one-$3\alpha,21$-diacetate	6.0 mg.[2]	2.0 (0.11%)[3]
Pregnane-$3\alpha,17\alpha,21$-triol-11,20-dione		44 (2.5%)
Hydrocortisone		5 (0.28%)
Cortisone		2.1 (0.11%)
Etiocholane-$3\alpha,11\beta$-diol-17-one & Δ^9-Etiocholene-3α-ol-17-one	absent	17.6 (0.98%)
11β-Hydroxyandrosterone and Δ^9-Androsten-3α-ol-17-one	3.0	4.4 (0.24%)
Etiocholane-3α-ol-11, 17-dione	2.5	9.0 (0.5%)
Etiocholane-3α-ol-17-one	26.0	absent
Androsterone	15.0	absent

[1] Values given are corrected to the equivalent of a urine collection of the cortisone administration period; calculations are based on the maximum amounts of crude material found.

[2] This value was determined by the formaldehydogenic method on the corticosteroid fraction.

[3] The percentages in parentheses are the conversion estimates of the administered cortisone equivalent to the urine available in this study (1.8 gm.).

DISCUSSION

The steroids isolated from both control and cortisone administration urines are listed in Table 2.

Similar to the results obtained in the hydrocortisone study (Burstein *et al.*, 1953), pregnane-3α,17α,21-triol-11,20-dione constituted the major steroid found in the corticosteroid fraction. Only a small quantity of pregnane-3α,11β,17α,21-tetrol-20-one was obtained. The conversion of cortisone to hydrocortisone occurred to the extent of 0.28 per cent. In a similar study Mason (1950) has indicated that hydrocortisone may be present following cortisone administration. The quantity of the excreted cortisone in the present study is of the same order of magnitude as that of the excreted hydrocortisone. This finding is similar to that which has been obtained following the administration of hydrocortisone (Burstein *et al.*, 1953). The pattern of the urinary corticosteroids excreted is the same whether cortisone or hydrocortisone is orally administered. The conversion of cortisone to etiocholane-3α, 11β-diol-17-one (the major 17-ketosteroid) and etiocholane-3α-ol-11,17-dione occurs to a lesser degree than the corresponding conversions following hydrocortisone administration (Burstein *et al.*, 1953). On the other hand, the extent of the conversion of cortisone to the pregnane derivatives appears to be larger than the corresponding conversion of hydrocortisone to these metabolites. During the cortisone administration period only a slight increase of 11β-hydroxyandrosterone and its dehydration product Δ^9-androstene-3α-ol-17-one was found. The difference between control and experimental urines was so slight that it is questionable whether cortisone is actually degraded in the human body to this 17-ketosteroid.

Although etiocholane-3α-ol-17-one and androsterone were isolated from the control urine, these steroids were not found in the cortisone urine. This finding is in accord with our studies on the influence on the excretion of these two 17-ketosteroids of high dosages of hydrocortisone (Burstein *et al.*, 1953).

Pregnane-3α,17α-diol-11,20-dione, which was isolated following cortisone and ACTH treatment by Lieberman *et al.* (1951) was not found in the present study.

SUMMARY

1. A total of 2.5 gm. of cortisone acetate has been administered to a man.

2. The following metabolites have been isolated and identified: pregnane-3α,11β,17α,21-tetrol-20-one as the diacetate, pregnane-3α,17α,21-triol-11,20-dione, hydrocortisone, cortisone, etiocholane-3α,11β-diol-17-one, Δ^9-etiocholene-3α-ol-17-one and etiocholane-3α-ol-11,17-dione.

3. No definitive evidence could be found for the conversion of cortisone to 11β-hydroxyandrosterone.

4. Etiocholane-3α-ol-17-one and androsterone which were present in the control urine in significant quantities, were completely absent from the urine following cortisone administration.

ADDENDUM

In a recent publication by Burton, Keutmann, Waterhouse (*J. Clin. Endocrinol. & Metabolism* **13**: 48. 1953), cortisone, hydrocortisone, pregnane-3α,17α,21-triol-11,20-dione and possibly pregnane-3α,11β,17α,21-tetrol-20-one were identified by paper chromatographic methods, following the parenteral administration of cortisone.

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THE EFFECT OF ADRENALECTOMY, SURGICAL TRAUMA, AND ETHER ANESTHESIA UPON THE CAPILLARY RESISTANCE OF THE ALBINO RAT*

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CAPILLARY leakage associated with widespread vascular dilatation has long been considered as a typical change after adrenalectomy. The regular finding of hemoconcentration (Athanasiu and Gradinesco, 1909) and of increased protein in the lymph (Cope, Brenizer and Polderman, 1942), and furthermore the observation that spreading of intradermally injected India ink is augmented after adrenalectomy in the mouse (Opsahl, 1949), have been regarded as evidence for the increased capillary *permeability*. That adrenalectomy may affect the capillary *resistance* as well was shown recently by Eichholz, Staab and Taugner (1951). Studying the blood pressure and the capillary resistance of rats during the first two weeks following adrenalectomy these authors found a decrease of the resistance from 76 to 46 cm. Hg as early as the third day. It is of interest that the change in the capillary resistance preceded the fall of the blood pressure by 1–2 days, and that both, hypotension and decreased resistance, could promptly be restored by administration of saline and Percorten (desoxycorticosterone glucoside in aqueous suspension).

While total removal of the adrenal glands followed by administration of cortical extract or isolated hormones may give information concerning the role of this organ in the maintenance of the capillary integrity, it is likewise possible that any change that may be found following this procedure may be due not only to the absence of the adrenal glands but also to the surgical trauma itself or even to the anesthetic. For this reason distinction should be made between early and late postoperative periods, the borderline between them being the time when the effects of the nonspecific factors become minimal or absent. In the early period, in addition, there is a fundamental disturbance in all functions of the organism with considerable vascular dilatation and capillary stasis but—in the rat provided with an increased salt supplement—compensatory readjustment soon occurs. This late postoperative state is more suitable for a study of

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any partial function of the body as well as for the testing cortical extracts or corticoids than is the early period.

The present work on the effect of adrenalectomy upon the capillary resistance was therefore preceded by a study of the non-specific effect of surgical trauma and anesthesia. The effect of adrenalectomy itself was studied during a period of 2–8 months.

METHOD AND PROCEDURE

Mature albino rats of the Sprague-Dawley strain and of both sexes with an average weight of 250 gm. and fed Purina Dog Chow were used. Twenty-four animals were adrenalectomized, 16 were subjected to sham adrenalectomy and 16 to ether anesthesia without surgical trauma. Deep ether anesthesia was given for 18 minutes. The adrenalectomy as well as the sham operation took 12 minutes. The length of incisions through skin and dorsal musculature as well as the number of stitches applied were standardized. In order to render the non-specific effects conspicuous these procedures were deliberately made in a more protracted and circumstantial manner. In the sham operation the adrenals were exposed—but not touched. Adrenalectomized rats were given 1% NaCl as drinking water. In addition 3 groups of rats, each consisting of 9 animals, were subjected to the same procedures (ether anesthesia, sham operation, adrenalectomy) with the purpose of studying the effect of ACTH and the corticoids. The animals receiving only ether anesthesia and those with sham operation were primed with the hormones before the respective intervention and for a certain period following it. The adrenalectomized rats were treated with the hormones in the late postoperative state.

The device used in these experiments for testing the capillary resistance consisted of a vacuum jar which was connected to an electrically powered suction pump on one direction and to a small plastic bell (of an inner diameter of 7 mm.) with a stopcock in the other direction. Degree of suction was measured by an intercalated mercury manometer. The skin of the abdomen of the animals was prepared by an electric hair clipper but it was not shaved. Shaving, in our experience, may irritate the skin and give rise to erroneous readings. A thin layer of liquid petrolatum applied to the skin before the testing provided a good surface for the suction cup to cling to and also facilitated viewing of the small petechiae. Suction was employed for 60 seconds. The least negative pressure given in cm. Hg. still capable of producing hemorrhage was considered as the value of the capillary resistance. As it was found that spasm of the precapillaries may lead to falsely high readings and thus interfere with the accuracy of the test, much care was taken to avoid or at least to recognize this condition. For this reason holding the animal in the palm of the glove hand was preferred, whenever possible, to restraining it by tieing it down to the board.

About one hundred healthy, untreated animals at different age levels have been tested for a period of over one year in order to determine the normal variation of the capillary resistance. It was found to range rather widely. The resistance was considered highest when no bleeding occurred even with a suction of 70 cm. (the average barometer reading in Omaha is approximately 730 mm.). It was then marked >70 cm. Healthy, well developed immature animals may show such a high resistance, the values being above 60 cm. Fully developed rats, sometimes all members of the same litter, may disclose a lower resistance (between 40 and 60 cm.), and apparently healthy animals may occasionally be found with exceptionally low levels (35–39 cm.). Though each animal tends to maintain its individual level during several months of observation with a fluctuation not exceeding ±5 cm., a general tendency to a gradual decrease with advancing age is noticeable. A capillary resistance under 27 cm. is considered to be definitely pathologic. Values of 35–27 cm. are looked upon as borderline. Thus the following arbitrary classification of the capillary resistance was found useful.

		Hg cm.
Normal	High	>70–61
	Medium	60–46
	Low	45–35
Intermediate		34–27
Pathologic	Moderately decreased	26–11
	Very low	10–0

The wide range of normalcy appeared to us rather enigmatic at first; its meaning, however, soon became evident in the course of this study.

RESULTS

Figure 1 shows typical patterns of capillary resistance change following the three types of intervention.

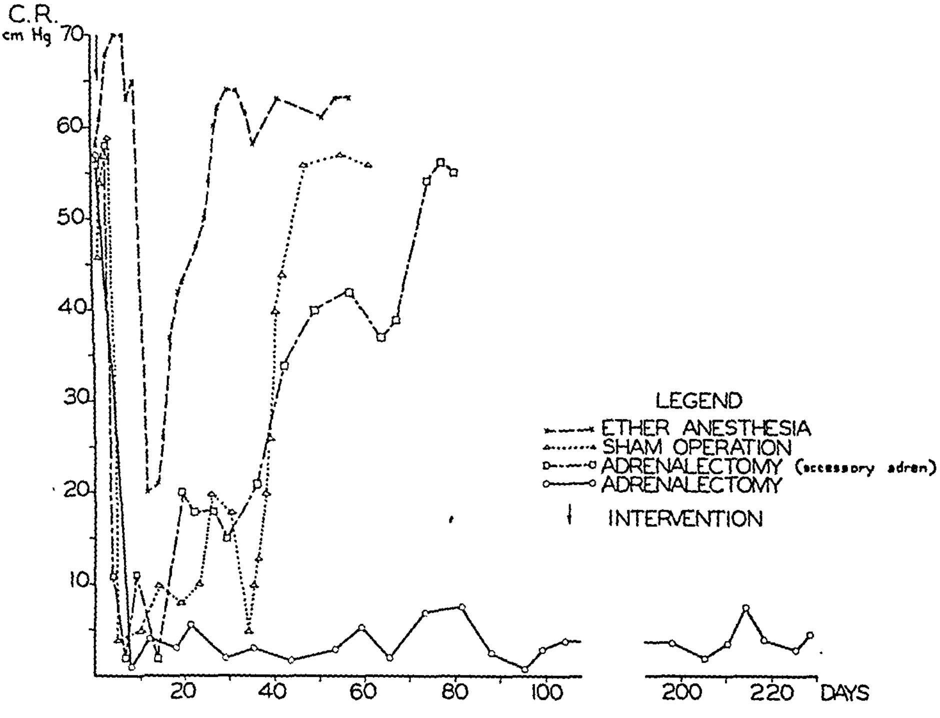

FIG. 1. Typical change in the capillary resistance of the rat following ether anesthesia, surgical trauma (sham adrenalectomy) and adrenalectomy. Change in the capillary resistance after adrenalectomy in a rat with accessory adrenal tissue shows no significant difference as compared to sham adrenalectomy.

Ether anesthesia. There is no change during the first 3–4 days although a tendency for the resistance to increase may often be noticeable. Between the fourth and the 12th day a more or less rapid decrease ensues and the resistance remains at pathologic levels for 1 to 6 days, whereafter it gradually rises and finally returns to its original value. The entire phenomenon, which may be termed "capillary crisis," occupies not more than 2 weeks. No other pathologic signs were observed during this entire period of capillary derangement. Wide individual variations were noted in onset, degree and duration of this "crisis." In three out of 16 animals the capillary resistance did not fall lower than the intermediate zone and in two strong male rats the crisis was entirely missed.

Surgical trauma (sham adrenalectomy). Here the typical crisis is more conspicuous than in the previous (ether control) experiments. Without exception the resistance began to decrease after the fourth day and then dropped in a rather abrupt manner and remained at pathologically low levels for a longer period of time than in the ether controls (14 days on the average). Recuperation was likewise more prolonged. The entire cycle took about one month. The critical drop of the resistance was sometimes preceded by a transient rise. The animals responded in a more uniform and marked way than when anesthesia was given without surgical trauma, although considerable individual variation in the capacity of restoring the resistance was commonly noted. During the first few postoperative days the animals were rather lively. Many of them, though not all, showed signs of general malaise and weakness during the second and third postoperative weeks. They did not eat well, became careless about their fur and their weight curves flattened. Some of them developed scattered small eczematous plaques.

Adrenalectomy. Being thus familiar with the response of the rats to anesthesia and surgical trauma, the effect of adrenalectomy was next studied. Twenty-four animals were adrenalectomized in deep ether anesthesia using the standardized technic as employed in the sham operation. Four of them died in the first days following operation, 10 died within two months, 9 survived for more than three months. In two of the 24 rats it was found that removal of the adrenals was not complete, in two other rats accessory adrenal tissue was revealed. The curve of the capillary resistance of these latter four animals was essentially the same as that seen after sham operation, though the restoration was more protracted. Figure 1 represents the behavior of the capillary resitance of one of the two animals with accessory tissue. The remaining 16 rats (the 4 that died within the first postoperative days are not considered here) showed a uniform pattern that was entirely different and is believed to be characteristic of the absence of the adrenals (Fig. 1.) The resistance decreased precipitously after the second day following removal of the glands and became, without exception, definitely pathologic after the 5th day in contrast to the more protracted slope in the curves shown by some of the sham operated animals. The essential difference, however, lies in the fact that whereas after sham operation the resistance returns to previous levels, it remained permanently low after adrenalectomy. The capillary resistance of several animals has been followed for 5 to 9 months after adrenalectomy and found to be consistently at or below 10 cm. Hg.

The effect of ACTH and Corticoids upon the pathologically low capillary resistance following adrenalectomy. The late postoperative period (2–8 months after operation), in which the animals are in a relatively balanced condition, having permanently low capillary resistance, seemed to be very suitable for checking hormonal effects. Figure 2 leaves no doubt about the effectiveness of cortisone.[1] In contrast, DOCA in high doses administered in aqueous[2] or oily[3] medium subcutaneously or intramuscularly was entirely ineffective. The slight rise in the curve occurring in some animals was

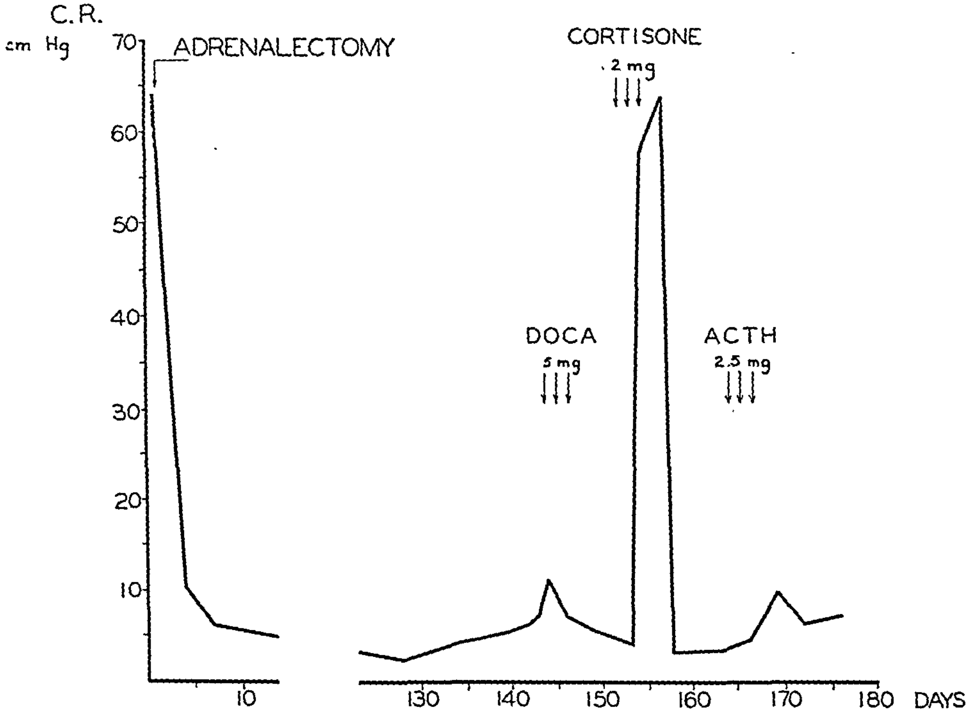

FIG. 2. Effect of cortisone, DOCA and ACTH on the capillary resistance of adrenalectomized rat in the late postoperative period.

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[1] 11-dehydro-17 hydroxycorticosterone 21-acetate (Cortone acetate, Merck).

[2] Desoxycorticosterone acetate aqueous macrosuspension (Bell Pharm. Co.).

[3] Desoxycorticosterone acetate in sesame oil (Cortate, Schering).

due to an edema of the skin caused by the huge doses of DOCA. ACTH[4] likewise showed no effect.

The effect of ACTH and Corticoids upon the capillary crisis elicited by surgical trauma and by ether anesthesia on normal (non-adrenalectomized) animals. Figures 3 and 4 show clearly evidence that cortisone is able to prevent the capillary crisis occuring after surgical trauma or ether anesthesia. All treated animals showed high normal resistance during the entire period of two weeks during which the capillary crisis might have been expected. In contrast all the animals primed with ACTH or DOCA disclosed the usual capillary response, i.e., these substances were unable to prevent the crisis. When, however, cortisone was withdrawn from the sham operated animals at a time when the resistance of the matched animals treated with ACTH or DOCA already returned to normal—a typical crisis set in. In case of anesthesia alone the resistance remained within normal values

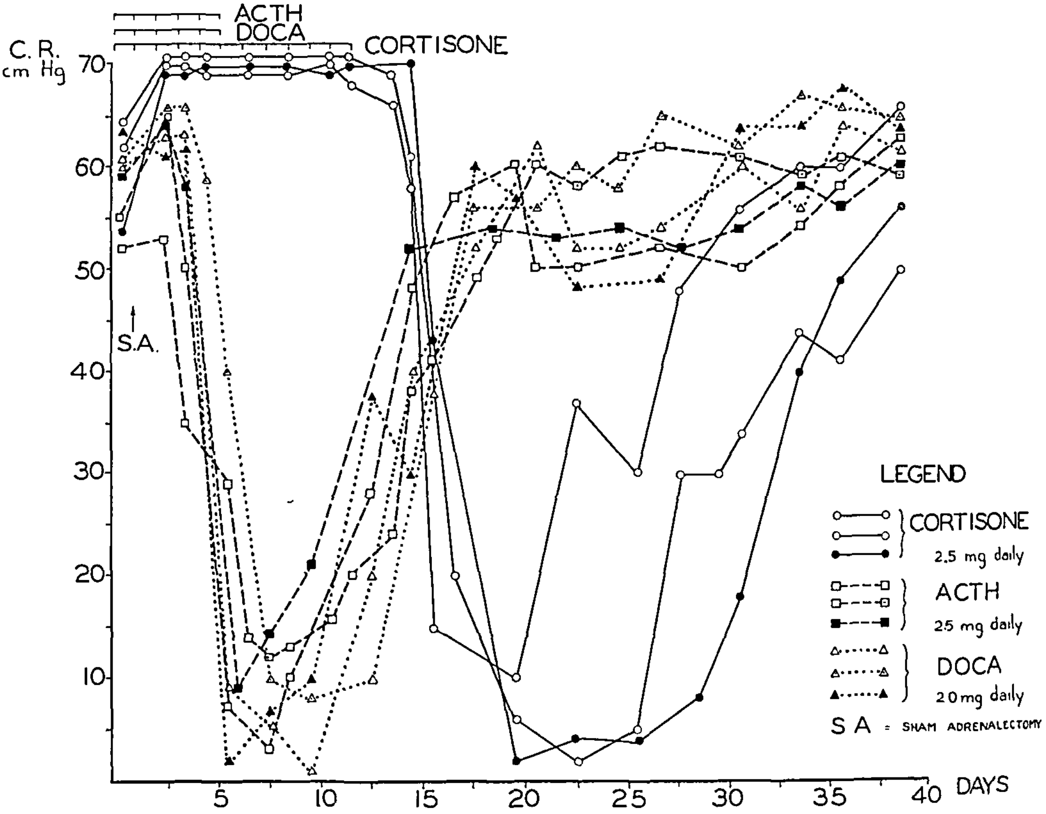

FIG. 3. Effect of cortisone, DOCA and ACTH on the "capillary crisis" due to surgical trauma. DOCA and ACTH were unable to prevent the crisis while cortisone was fully effective. Withdrawal was followed by a typical "secondary capillary crisis."

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[4] Lyophilized adrenocorticotropic hormone (ACTHAR, Armour Lab.).

after cortisone was discontinued. However, there was a small indentation in the curve suggesting a rudimentary form of a crisis.

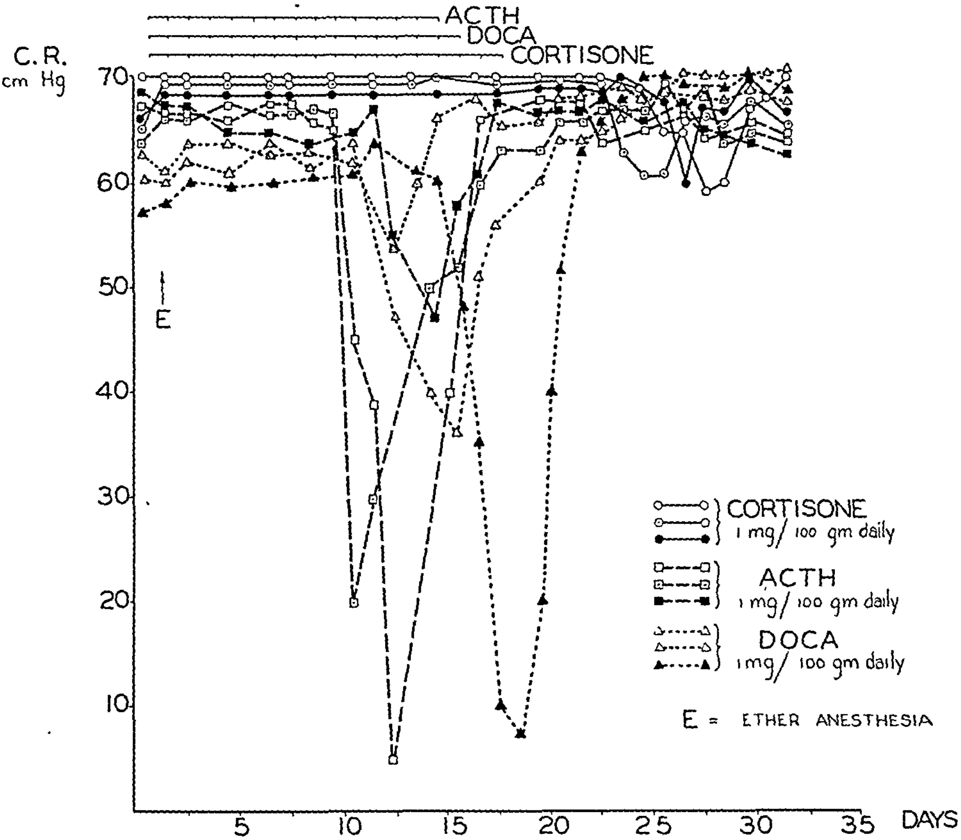

FIG. 4. Effect of cortisone, DOCA and ACTH on the capillary crisis elicited by ether anesthesia. In these experiments the rats were kept in an atmosphere containing 6 vol. % ether for 90 minutes. Cortisone was fully effective in preventing the capillary crisis whereas DOCA and ACTH failed to do so. Rudimentary form of "secondary capillary crisis" after withdrawal of cortisone.

DISCUSSION

The behavior of the capillary resistance after adrenalectomy studied in a long range experiment of several months' duration definitely suggests that the maintenance of the normal capillary resistance is a function of the adrenal cortex. Administration of ACTH, cortisone, and DOCA in the late postoperative phase seem to indicate that cortisone is involved in this function. Eichholz *et al.* (1951), as mentioned above, attribute the same role to DOCA.

The present study had already been completed when the paper of Eichholz *et al.* became available to us. Thus without repeating their experiments it can only be stated that DOCA was found ineffective under our experimental conditons. It is presumed that the explanation for this discrepancy may be found among the following factors.

	Eichholz et al.	*Authors*
Observation made in period	early postoperative	late postoperative
Duration of observation	two weeks	up to 8 months
Range of capillary resistance tested	76–46 cm. Hg (Within Normal zone)	70–0 cm. Hg (including Intermediate and Pathologic zones
Administration of NaCl	parenterally (mostly) with DOCA whenever it was tested	Constant oral supply during entire period of observation
Preparation used	Percorten, aqueous (desoxycorticosterone glucoside)	Desoxycorticosterone acetate aqueous macrosuspension (Bell Pharm Co.); In sesame oil (Cortate, Schering)

Since the early observation of Leriche upon the "maladie post-opératoire" and its recognition as a pathologic entity (an example of the alarm reaction by Selye) the systemic changes caused by traumatic injury have been the subject of considerable interest and innumerable studies. Nevertheless little is known about the behavior of the capillaries in this condition and the available data are, in addition, rather conflicting. On the one side there stands the still debated question whether or not a traumatic shock may produce capillary *leakage* at a distance from the site of the injury (pro: Selye, contra: McMichael, 1945; Nobel and Gregersen, 1946)—on the other side the observation of Cole, Shaw and Fraser (1950) suggesting that trauma may lead to a decrease of the permeability. Concerning capillary resistance only two data are available (Ungar, 1944; Robson *et al.*, 1950). both indicating an *increase* after tissue damage. No reference whatsoever could be found in this relation concerning ether anesthesia.

In view of these data in the literature the capillary crisis found in our experiments after relatively minor injuries such as sham adrenalectomy or anesthesia seemed rather enigmatic at first glance. When one compares these curves, however, with those of the adrenalectomized animals and considers the effect of cortisone in all these conditions, these findings become more comprehensible.

If the adrenal cortex governs the capillary resistance and if cortisone is a mediator of this function, the critical drop of the capillary resistance following stress and the period of low resistance that ensues thereafter must necessarily mean either that the stimulating effect of the pituitary upon the adrenal was lacking during this time or that the adrenals were *not* responsive. As ACTH failed to prevent the capillary crisis (note: The animals were not adrenalectomized) whereas cortisone was fully effective the second possibility must be thought of. Since it is known that the demand of the organism for corticoids is increased during stress the explanation may be offered that this unresponsiveness of the adrenal and, hand in hand with it, the capillary crisis, are expressions of a transient hypofunction or depletion. (The term "exhaustion" is deliberately avoided to prevent confusion with Selye's concept of "state of exhaustion.")

Details of an analysis of this remarkable condition will soon be published on the basis of studies that have been made with various other forms of stress. The phenomenon of the "secondary capillary crisis" occurring after discontinuance of cortisone on animals in which the primary crisis due to a stress was prevented by it will also be the subject of a special study.

SUMMARY

The change of the capillary resistance of the albino rat following adrenalectomy was studied for a period of 2 to 8 months. In order to rule out the possible effects of surgical trauma and anesthesia (factors necessarily involved with the adrenalectomy) upon the capillary resistance, some groups of rats were sham operated in ether anesthesia or subjected to the anesthesia alone.

After *sham adrenalectomy* the capillary resistance changed in a typical way showing a pattern with 4 successive phases: (1) a more or less pronounced increase in the first days; (2) a critical drop at the end of the first or in the course of the second week; (3) a state of pathologically low resistance lasting about two weeks; (4) a period of recuperation. The entire phenomenon—it may be termed "capillary crisis"—takes about one month. Though individual variations were often noticed, on the whole, the response of the animals was remarkably uniform.

Following *ether anesthesia* the same pattern was observed, mostly in somewhat telescoped and less conspicuous form. Individual variations were more common.

After *adrenalectomy* only the second and third phases of the above pattern were seen, i.e., the initial rise was absent and the capillary resistance remained at low levels during the entire period of observation (up to 9 months). In the presence of accessory adrenal tissue the animals responded in the same way as after sham operation, although restoration was more protracted.

Since the first phase of the capillary crisis (initial rise of the resistance before its critical drop) in the sham operated or merely anesthetized animals may often be absent or easily missed, it is obvious that during the first 3 to 4 weeks following operation there is no significant difference between adrenalectomized and sham operated animals as far as capillary

resistance is concerned. In view of this observation as well as of the possibility that hemodynamic changes ensuing during the first weeks after removal of the gland may also influence the capillary integrity, it must be inferred that the late postoperative period is, in fact, more suitable that the early period for a study of the effect of adrenalectomy upon the capillary resistance as well as for testing hormonal or other preparations.

A study in this late postoperative period showed that cortisone is able to restore the capillary resistance promptly. ACTH and DOCA (both aqueous and oily) were found ineffective. Cortisone given to sham operated or merely anesthetized animals before the procedures prevented the capillary crisis. DOCA and ACTH failed to do so.

These findings suggest that the adrenal cortex is intimately connected with the resistance of the capillaries and that cortisone seems to be involved in this function.

The explanation is offered that the "capillary crisis" found after surgical trauma or anesthesia may be an expression of a transient hypofunction or depletion of the adrenal cortex.

Acknowledgment

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THE EFFECTS OF ANDROGEN AND ESTROGEN IN CASTRATED AND HYPOPHYSECTOMIZED C_3H MICE

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INTRODUCTION

ESTROGEN-ANDROGEN antagonism may be defined as the suppression or modification of the effect of one hormone on an end-organ or tissue by the opposite sex hormone. The phenomenon has been demonstrated in many experiments (review, Burrows, 1949), but it is not clear to what extent antagonism occurs at the site of action on the end-organ, or to what extent it is brought about indirectly through changes in pituitary or gonadal function. The experiments of Moore and Price (1932) seemed to indicate that no estrogen-androgen antagonism existed except as mediated through the influence of the gonadal hormones on pituitary gonadotropic function. Martin (1951), on the other hand, has stated that estrogen-androgen antagonism probably holds for any tissue affected by either hormone, and that inhibitions are due to direct antagonistic action in the tissues.

The present study was designed to indicate the extent of estrogen-androgen antagonism occurring in animals from which pituitary and gonadal influences were eliminated, at a constant dose level of the two sex hormones.

METHODS

A total of 160 C_3H strain mice were used in the experiment. The animals were singly housed, and water and Purina Fox Chow were provided ad libitum. Hypophysectomy by the method of Thomas (1938), and castration were performed in 140 of the mice. Completeness of hypophysectomy was evidenced by a continuing loss of weight in every animal used in the experiment, absence of visible pituitary tissue on examination with a dissection microscope at post-mortem, and absence of recognizable pituitary tissue in serial sections of the decalcified sella turcica in 4 representative animals.

Injected hormones included α-estradiol, diethylstilbestrol, and testosterone propionate, hereafter referred to as estradiol, stilbestrol, and testosterone, respectively. The total daily hormone dose was injected subcutaneously in 0.03 ml. of oil, in one of 3 locations varied in regular rotation.

The mice were 3 to 4 months old at operation. Injections were begun 31 to 35 days after operation, and continued daily for 14 days. Vaginal smears were taken daily by lavage in all females during the injection period. The mice were killed with ether on the

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day following the last injection. Organ and body weights were obtained from each animal, and tissue sections and mammary whole mounts were prepared from 2 or more animals in each group.

RESULTS

Preliminary experiment to determine minimum doses of hormones producing maximal effect in castrated and hypophysectomized mice.

It is well known that the relative dosage of androgen and estrogen may determine whether antagonistic, cooperative, or independent effects will be produced in a given tissue (Burrows, 1949). Selection of doses for the present experiment was made as follows. Weights of seminal vesicles with their retained secretion in 6 castrated and hypophysectomized male mice, subjected to graded doses of testosterone injected daily for 14 days, indicated that the minimum dose producing a maximum increase was about 20 μg. daily. Eight castrated and hypophysectomized female mice were injected with graded doses of stilbestrol, and six were injected with graded doses of estradiol, daily for 14 days. The minimum dose of stilbestrol producing a maximal increase in uterine weight was 0.08 μg., and the corresponding dose of estradiol was 0.01 μg.

When testosterone was used in female mice, the dose as determined above was arbitrarily doubled, to 40 μg. daily. The dose of estradiol given to male mice was increased to 0.1 μg. daily. The dose of stilbestrol used in the male mice could be increased only to 0.1 μg. daily, because larger doses had a toxic effect.

The main experiment was arranged as follows: there were 70 mice of each sex, divided into 7 groups of 10. One group of each sex was intact, and the remainder were castrated and hypophysectomized; of the latter, one group of each sex was injected with corn oil without any hormone, and one group of each sex with each of the following: testosterone, estradiol, stilbestrol, testosterone plus stilbestrol, and testosterone plus estradiol.

Body weight (Tables 1 and 3)

The males weighed more than the females. All of the mice which were

TABLE 1. AVERAGE ORGAN AND BODY WEIGHTS FROM INTACT FEMALE MICE AND FROM THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED FEMALE MICE. TEN MICE IN EACH GROUP

Numbers are means in mg. ± standard deviation

Organ	Intact	No horm.	Testosterone	Stilbestrol	Estradiol	Stilb.-test.	Estra.-test.
Adrenal	2.76±0.33	0.99±0.10	0.88±0.10	0.90±0.15	0.87±0.14	0.76±0.07	0.79±0.15
Heart	101±7	50±3	52±4	48±2	48±3	49±2	50±4
Kidney	168±17	75±6	103±10	73±5	71±6	97±7	103±10
Liver	1397±127	967±92	1142±81	856±54	865±150	972±102	1081±71
Spleen	80±12	41±9	45±4	41±6	38±5	39±8	36±3
Thymus	35±3.4	26±3.5	14±1.7	29±3.8	27±4.7	10±2.5	19±3.6
Uterine Horn	43±9.6	5.3±0.6	21±2.9	30±4.4	21±3.1	19±3.4	18±2.5
Body (Autopsy)	25.0 Gm.	16.3	17.6	16.0	15.8	16.0	17.1

TABLE 2. STATISTICALLY SIGNIFICANT DIFFERENCES IN ORGAN WEIGHTS BETWEEN THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED FEMALE MICE, LISTED ACCORDING TO HORMONES INJECTED. WHEN THE MEAN ORGAN WEIGHT OF ONE OF THE HORIZONTALLY LISTED GROUPS IS LARGER THAN THAT FROM A VERTICALLY LISTED GROUP, AND P IS LESS THAN 0.01, THAT FACT IS INDICATED BY ++; IF P IS LESS THAN 0.05, BY +. MINUS SIGNS INDICATE THE OPPOSITE RELATIONSHIP

Organ	Animal groups	Animal groups					
		No horm.	Test.	Stilb.	Estra.	Stil.-Test.	Extra.-Test.
Adrenal	No horm.		–			– –	– –
	Testost.	+				– –	
	Stilb.					– –	
Kidney	No horm.		++			++	++
	Stilb.		++			++	
	Estra.		++				++
Liver	No horm.		++	– –			+
	Test.	– –		– –	– –	– –	
	Stilb.	++	++			+	
	Estra.		++				++
Spleen	Test.				– –		– –
Thymus	No horm.		– –			– –	– –
	Test.	++		++	++	– –	++
	Stilb.		– –			– –	
	Estra.		– –				– –
Uterine Horn	No horm.		++	++	++	++	++
	Test.	– –		++			–
	Stilb.	– –	– –			– –	
	Estra.	– –					–

TABLE 3. AVERAGE ORGAN AND BODY WEIGHTS FROM INTACT MALE MICE AND FROM THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED MALE MICE. TEN MICE IN EACH GROUP

Numbers are meanns in mg. ± standard deviation.

Organ	Intact	No horm.	Testosterone	Stilbestrol	Estradiol	Stilb.-test.	Estra.-test.
Adrenal	1.76±0.12	0.82±0.07	0.94±0.15	0.77±0.08	0.77±0.09	0.80±0.09	0.77±0.11
Heart	118±5	53±3	54±2	53±3	56±3	54±4	53±4
Kidney	227±16	89±5	110±7	95±6	99±9	114±10	115±10
Liver	1605±119	917±84	1089±65	956±49	1032±110	964±90	1022±106
Spleen	67±7	34±3	42±3	47±8	48±4	36±5	38±5
Thymus	30±5.0	22±4.9	25±2.1	20±2.2	24±3.4	15±3.3	14±1.6
Seminal vesicle	66±9.9	4.3±0.6	23±3.2	4.1±0.5	4.9±1.0	17±2.4	19±2.2
Body (autopsy)	28.1 Gm.	18.1	18.3	18.7	18.6	17.5	16.3

operated on lost weight, from 13 to 32% between operation and autopsy. Differences between groups of castrated and hypophysectomized mice are not statistically significant. All the intact mice gained weight during the same period.

Vaginal Smears

Castrated-hypophysectomized mice receiving no hormone injections had unvarying, scanty vaginal smears in which there were only a few cells, mostly leukocytes, and little or no mucus. Estradiol or stilbestrol produced an abundance of cornified cells after the third or fourth day.

Testosterone produced increased mucus, "mucified" cells (Fig. 3), and leukocytes after the third day, but no cornified cells were seen.

When both testosterone and estradiol were given, the picture was not distinguishable from that produced by testosterone alone.

TABLE 4. STATISTICALLY SIGNIFICANT DIFFERENCES IN ORGAN WEIGHTS BETWEEN THE SIX GROUPS OF CASTRATED AND HYPOPHYSECTOMIZED MALE MICE, LISTED ACCORDING TO HORMONES INJECTED. SYMBOLS AS IN TABLE 2

Organ	Animal groups	Animal groups					
		No horm.	Test.	Stilb.	Estra.	Stil.-Test.	Estra.-Test.
Adrenal	No horm.		+				
	Test.	−		−	−	−	−
Kidney	No horm.		++		+	++	++
	Stilb.		++			++	
	Estra.	−	+				++
Liver	No horm.		++		+		+
	Test.	−−		−−		−−	
Spleen	No horm.		++	++	++		
	Test.	−−				−	
	Stilb.	−−				−−	
	Estra.	−−					−−
Thymus	No horm.					−−	−−
	Test.			−−		−−	−
	Stilb.		++			−−	
	Estra.						−−
Seminal Vesicle	No horm.		++			++	++
	Test.	−−		−−	−−	−−	−
	Stilb.		++			++	
	Estra.		++				++

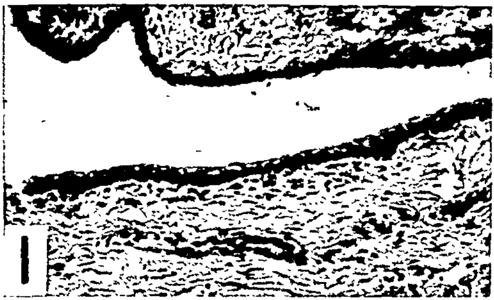
When testosterone was combined with stilbestrol, there was a varying predominance of cornified or mucified cells from day to day in a given animal. Both types of cell were seen, along with mucus and leukocytes in most of the smears (Fig. 4).

EXPLANATION OF FIGURES 1–8

Photomicrographs of tissues from castrated and hypophysectomized female mice. H. and E. stain. ×150.

FIGS. 1–5 Vaginae. 1) No hormone given. 2) Stilbestrol. Epithelium is similar to that in intact mice. 3) Testosterone. 4) Stilbestrol and testosterone. 5) Estradiol and testosterone.

FIGS. 6–8 Uteri. 6) No hormone given. The magnification appears different from that in Figs. 7 and 8, but it is the same. 7) Stilbestrol. 8) Stilbestrol and testosterone. The uteri from mice given testosterone alone, and those given estradiol, or estradiol and testosterone, were similar to Fig. 8.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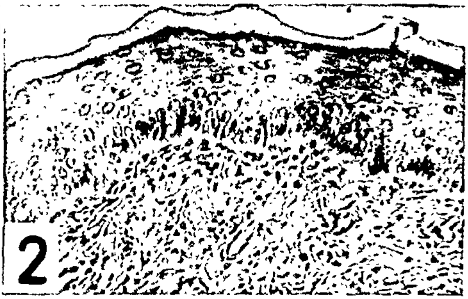
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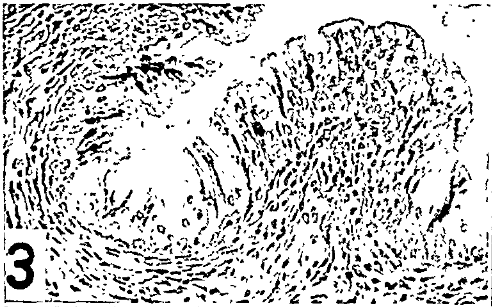
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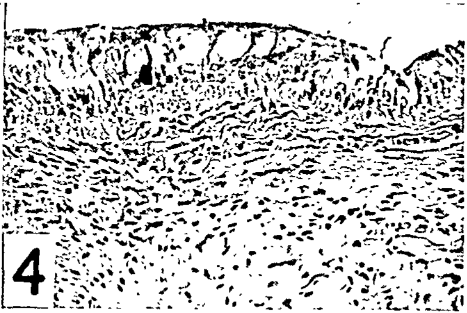
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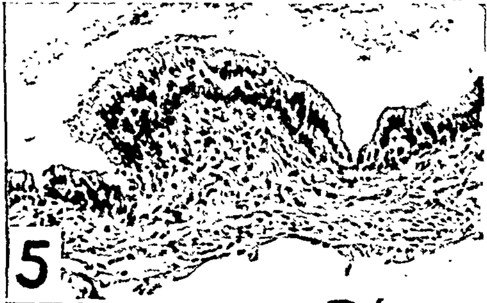
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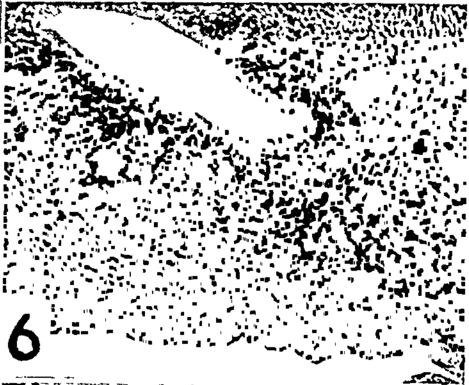
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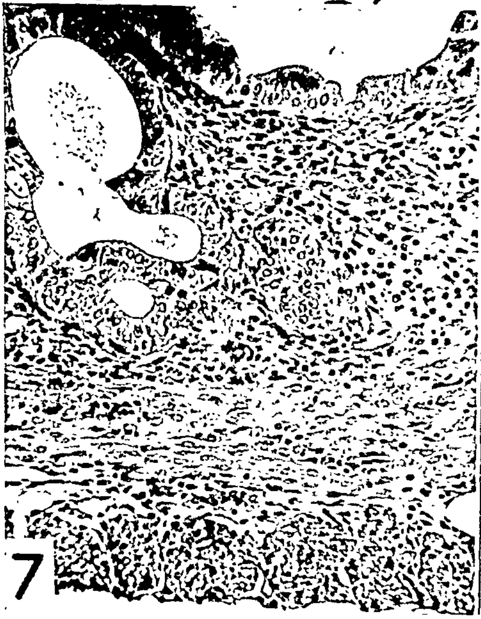
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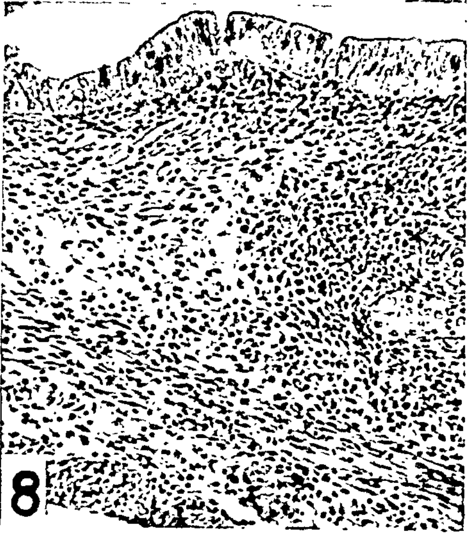
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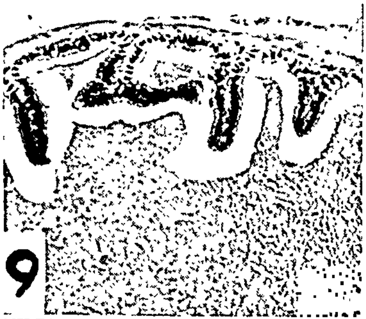
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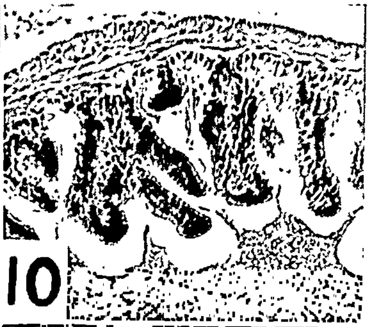
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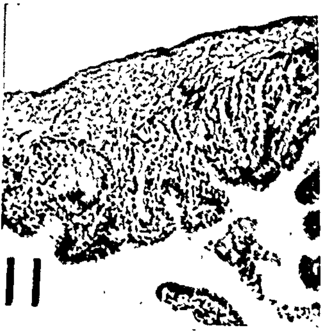
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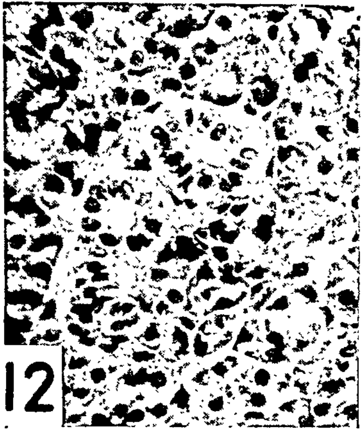
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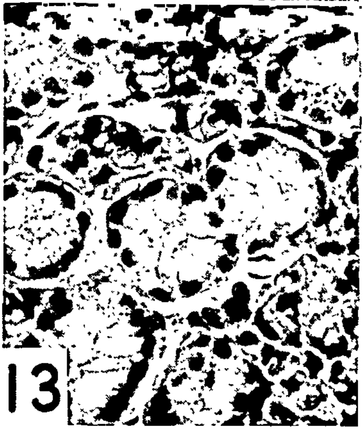
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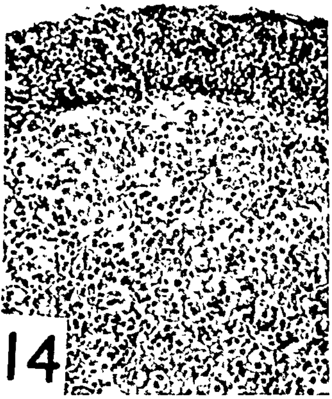
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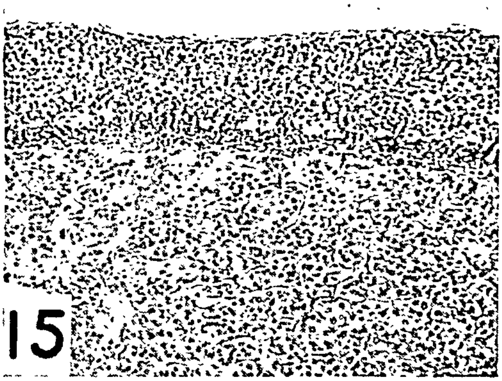
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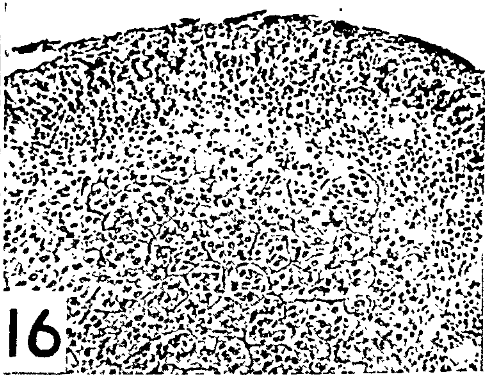
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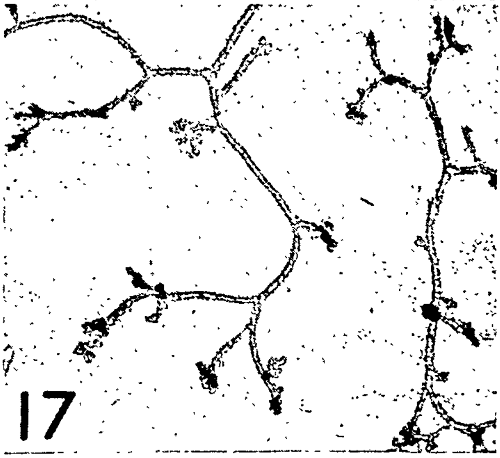
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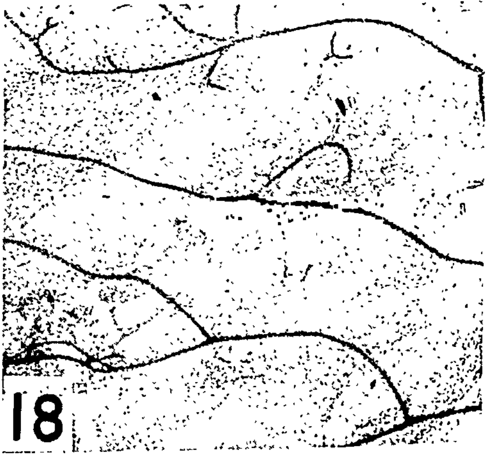
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Changes in organ weights (Tables 1–4)

Uterine horn. The uterus underwent severe atrophy after castration and hypophysectomy. It was restored to nearly $\frac{3}{4}$ normal size by injections of stilbestrol, and to about half normal size by either estradiol or testosterone. A significant inhibition of the effects of either stilbestrol or estradiol was found when testosterone was added; in fact, when both estrogen and androgen were given, uterine weights were less in each case than when either was given alone. The inhibitory effect appeared to be mutual.

Seminal vesicles. These were weighed without loss of secretion, and judging by the histology (Figs. 9–11), the differences in weight are mainly due to differences in the amount of secretion present. Pronounced atrophy as well as decreased secretion occurred after operation. The injection of testosterone produced secretion and a gain in weight to about $\frac{1}{3}$ normal size. This effect was partially inhibited by either estradiol or stilbestrol, neither of which had any effect when given alone.

Adrenals. The sections (Figs. 14–16) show that differences in adrenal weights are mainly due to changes in the cortex. The findings were different in the two sexes: In the female, changes were always in the direction of smaller glands when any hormones were given, and there was more atrophy when both hormones were given than in the other groups. In the male, the only significant change from the untreated controls was a slight increase in weight when testosterone was given alone, which was apparently completely inhibited by simultaneous administration of either stilbestrol or estradiol.

Kidneys. There was a reduction in weight of 55% in the female and 61% in the male after operation. An increase in weight was produced in either

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Explanation of Figures 9–18

Photomicrographs, H. and E. stain. ×150 or 200.

Figs. 9–11. Seminal vesicles from castrated and hypophysectomized male mice. 9) Given testosterone. 10) Testosterone and estradiol. 11) Estradiol. Seminal vesicles from mice given stilbestrol and from those not given any hormones closely resemble Fig. 11.

Figs. 12 and 13. Submaxillary glands from castrated and hypophysectomized female mice. 12) Given stilbestrol. This appearance was found in all groups except intact males and mice given testosterone. 13) Stilbestrol and testosterone. Testosterone alone and testosterone plus estradiol gave the same picture as Fig. 13.

Figs. 14–16. Adrenal glands from castrated and hypophysectomized female mice. 14) Given testosterone. 15) Stilbestrol. 16) Testosterone and stilbestrol.

Figs. 17 and 18. Mammary whole mount preparations ×30. 17) Intact female mouse. 18) Castrated and hypophysectomized female mouse given stilbstrol; those given the other hormones or no hormone had the same appearance.

sex by testosterone, and this effect was not inhibited by estrogen. Stilbestrol alone had no effect, but there was some increase in weight of the male kidney when estradiol was given.

Liver. Liver weight was increased by testosterone, and this effect was inhibited by stilbestrol, but not by estradiol, which by itself produced an increased weight in the male.

Thymus. Weight was decreased in the female by testosterone, and this was unchanged when stilbestrol was added, but the decrease was less if estradiol was added. In the male there was a decreased weight when two hormones were given together, but no effect from any of the three given separately. These changes were not correlated with the differences in adrenal weights.

Spleen. In the male, weights were increased when estrogen or androgen were given alone, but were unaffected when both were given. In females there was little change.

Heart. The heart weight was remarkably constant and unaffected by sex hormones.

Histology

After operation, there was atrophy of nearly all tissues, with a decrease of cytoplasm and closer packing of cells.

Vagina (Figs. 1–5). Following operation there was pronounced atrophy of the entire structure. Epithelium was reduced from about 8 layers of cells to two layers without cornification. When estradiol or stilbestrol was given, the epithelium returned to approximately normal, but the rest of the vaginal wall remained atrophic. Injections of testosterone produced a vaginal mucosa 3 to 4 layers thick, with large pale-staining cells, and with mucus being discharged into the lumen. When estradiol was given with testosterone, the picture was similar to that with testosterone alone. When testosterone and stilbestrol were combined, the pattern varied, with the deeper layers of the mucosa resembling the stratified squamous, and the layer nearest the lumen mucified in most areas.

Uterus (Figs. 6–8). The extreme atrophy occurring in this organ after castration and hypophysectomy is but little more than that occurring after castration alone. There was considerable restoration of all layers after injection of estrogen. Many endometrial glands were formed when stilbestrol was given. This effect was diminished, and muscular development was less evident if testostrerone was added to stilbestrol. Estradiol had less effect on musculature, and glands were smaller than those found after stilbestrol injections. Uteri from animals injected with testosterone, or testosterone and either estrogen were very similar histologically, and are well represented in Figure 8.

Seminal vesicles (Figs. 9–11). The appearance in those mice given no hormone or either estrogen is the same (Fig. 11). Testosterone caused the organ to become distended with secretion, and this effect was decreased when estrogen was added.

Prostate and coagulating glands. The flattened eipthelial cells seen in mice given no hormone after operation were changed to larger, cuboidal cells by testosterone, but were apparently unaffected by estrogen. There was no squamous metaplasia, and estrogen did not modify the effect of testosterone. Squamous metaplasia has been reported in these structures after doses of estrogen considerably larger than used here (Thorborg, 1948).

Submaxillary glands (Figs. 12, 13). Sexual bimorphism in this organ was originally described by Lacassagne (1940). In the present experiment, mice operated on all had the female type of ductule (Fig. 12), except those given testosterone, which all had the male type (Fig. 13). Ductules of the latter were not modified when estrogen was added to testosterone.

Kidneys. The number of Bowman's capsules having cuboidal cells in the parietal layer is increased in the castrate male mouse by injection of testosterone (Crabtree, 1941). In the present experiment it was not possible to find significant differences in the number of such capsules in the various groups, although higher numbers were found in males given testosterone.

Adrenals (Figs. 14–16). All adrenals from animals operated on showed atrophy of the cortex, and this feature was not conspicuously modified by any of the hormones given. The cortex in the male was essentially unchanged in appearance by the various injections. In the female after operation there was a concentration of small dark cells in the inner part of the cortex, about 8 layers thick, representing the atrophied x-zone. This area was not distinguishable in females given testosterone, with or without estrogen, but was present in all others.

The female mice given testosterone with either estrogen showed an uneven basophilic staining of nuclei in both cortex and medulla (Fig. 16). This may represent more rapid atrophy or degeneration, and is correlated with the greater loss of adrenal weight in these groups.

Areas of "brown degeneration," a deposition of ceroid pigment, were found in most of the female adrenals, in the inner part of the cortex. They were most prominent in the group given stilbestrol (Fig. 15).

Other organs. Sections were studied from the following organs without finding significant differences between the various groups of castrated and hypophysectomized animals: liver, pancreas, spleen, sublingual gland, thyroid, parathyroid, trachea, esophagus, thymus, lung, heart. stomach, duodenum, ileum, colon, and bladder.

Mammary whole mounts (Figs. 17, 18)

The mammary glands were removed from the skin of each animal and examined, and complete preparations were made from two animals of each group. There was no development of the mammary ducts in any of the animals which had been castrated and hypophysectomized. Examples of estrogen-androgen antagonistic effects on the mammary gland in intact or castrated animals have been reported (Smelser, 1933; Arbelger and Huseby, 1951), and may have involved pituitary suppression as part of the mechanism; however, the level of pituitary factor necessary for mammary growth might be unaffected by sex hormones, though essential for their action.

DISCUSSION

True hormone antagonism might be defined as a situation where a measured characteristic of a target organ is affected in one direction by one hormone, and is affected only in the opposite direction by addition of the other hormone. Examples of such antagonism in the present study were observed in the amount of seminal vesicle secretion, the weight of the liver, the weight of the thymus in the female, and the extent of the x-zone in the female adrenal cortex.

Other types of effect observed included instances where both sex hormones produced alterations in the same direction, but where one nevertheless inhibited the effect of the other, as in development of the endometrium, uterine weight, and splenic weight in the male, A site where different, but not opposite, effects were exhibited separately by the two sex hormones was the vaginal mucosa. Examples of places where only one hormone showed activity were the ductules of the submaxillary gland, and the weight of the kidney (testosterone versus stilbestrol). Finally, in two instances the two hormones together had a synergistic effect which neither had alone, on thymus weight in the male and adrenal weight and histology in the female.

The idea that all tissues will respond to one sex hormone if they are affected by the opposite one could not be proved or disproved unless an extensive dose range of both hormones were tested; in the range of dosage near physiological, which we have used, this conception did not apply. A theory of universal estrogen-androgen antagonism is not supported by our data.

Pituitary function may affect in some degree the response of various target organs to injected sex steroids, but the essential agreement between the results in this experiment and those reported from non-hypophysectomized animals implies that pituitary hormones are not indispensable to sex steroid action on the target organs studied, except the mammary gland. The latter did not respond to injected steroids after hypophysectomy.

SUMMARY AND CONCLUSIONS

A study has been made in 140 castrated and hypophysectomized C_3H male and female mice, and 20 intact controls, of the effects of injected testosterone propionate, and diethylstilbestrol or α-estradiol, separately and in combination. Injections were begun 31 to 35 days after operation and continued daily for 14 days. Observations were made of vaginal smears, body weight, organ weights, histology, and mammary whole mounts.

The results indicated that antagonistic effects of simultaneously administered estrogen and androgen in the castrated and hypophysectomized mouse are evident, but are not predominant; there are also independent, unlike effects of the two hormones on the same tissue, sites where only one hormone is effective at the given dose level, similar effects by both hormones on the same tissue, and synergistic effects not seen with either hormone alone. The similarity of these findings to those reported in intact or castrated animals suggests that the pituitary does not play a determining role in the mechanism of action of the sex hormones on the target organs studied, except the mammary gland.

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EFFECTS OF CORTICOSTEROIDS ON URINARY FORMALDEHYDOGENIC CORTICOIDS AND 17-KETOSTEROIDS IN NORMAL AND SUBTOTALLY HEPATECTOMIZED RATS[1]

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URINARY outputs of 17-ketosteroids (17-KS) and reducing (R) or formaldehydogenic (F) corticoids (C) are used as measures of adrenal cortical function. However, they are not readily interpretable. Under different conditions, urinary 17-KS and FC can vary in the same sense or independently (Venning, 1950). Among factors contributing to this variability of relationship, the nature of the steroids undergoing metabolism is perhaps the most important. Thus, in human beings (Venning, 1950; Polley and Mason, 1950) and in rats (del Greco, Masson and Corcoran, 1952), only C-17-hydroxylated corticosteroids act as 17-KS precursors; the extent to which injected corticosteroids appear in urine as FC varies widely (Fajans, Louis and Conn, 1951 a). Among other variables are the state of esterification, the route of administration and the level of hepatic function (Fajans, Louis and Conn 1951 b; Engstrom *et al.*, 1951; Bongiovanni and Eisenmenger, 1951). The aim of this study was to test the significance of these factors on absolute and relative outputs of urinary 17-KS and FC.

A method, previously described for the measurement of urinary 17-KS in the rat (del Greco, Masson and Corcoran, 1952), was adapted which depends in principle on giving large doses of a test compound over a short space of time, so that effects of endogenous hormones are obscured. The variables tested in these experiments are (a) the nature of the compound, (b) its esterification, (c) oral as compared with subcutaneous administration, and (d) the effect of subtotal hepatectomy.

PROCEDURE

Female albino rats (150 to 250 gm. body weight) were divided in groups

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of at least 4 animals. Each rat was placed separately in a metabolic cage; the animals were maintained on Purina Fox Chow; 1% NaCl was substituted for drinking water in order to increase urine flow.

The pretreatment control period of 4 days enabled the animals to accustom themselves to their new environment; they were then placed on real or simulated treatment for 5 days; this was followed by a recovery period of 4 days; lack of certain steroids sometimes forced us to shorten the treatment period by 1 or 2 days. In experiments involving sub-total hepatectomy, the operation was done on the last pretreatment day.

The corticosteroids tested were cortisone and hydrocortisone, each as alcohol and acetate, and the acetates of corticosterone, dehydrocorticosterone and desoxycorticosterone; free testosterone was injected as a means of validating the 17-KS method. These steroids were given once a day in doses of 10 mg. in water suspension; the subcutaneous dose was contained in a volume of 0.2 cc. and the oral dose was given by gavage in a volume of 1.0 cc.

Urine was collected daily; 17-KS determinations were done on individual Merthiolate-preserved samples by a method of Drekter *et al.* (1952); determinations of pH 1 extractable FC (Corcoran and Page, 1948) were done on chloroform-preserved samples pooled daily from each group. The extraction stages of the procedures were completed on the day of collection; the extracts were then accumulated for simultaneous final colorimetric analyses. The urine samples were grossly free of feces and food.

This manner of handling the samples is important as concerns FC. In a former study (Masson *et al.*, 1952) urines of individual rats were pooled after addition of chloroform and accumulated at 10° C. to a volume of 300 cc., so that several days elapsed before acidification and extraction. Mean FC output of normal rats by this procedure was 0.5 μg. daily. By the present procedure, the mean of 14 observations (urines from 36 individuals) is 11.2 μg. daily, the standard deviation ±3.7 and the standard error of the mean ±1. The effect of spleen β-glucuronidase (Viobin) was examined by comparison of FC estimates in 5 pools of fresh rat urine; 2 were extracted at pH 1 without incubation with the enzyme and yielded values of 4 and 3 μg. daily; the 3 samples extracted at pH 1 after 3 days incubation with the enzyme (procedure 1 of Dustan, Mason and Corcoran, 1953) gave negligible yields in 2 cases and value of 1.5 μg. in the other. The difference in yield between the directly extracted and enzyme incubated samples indicates that there had been destruction of FC during the time of incubation; similar destruction which may be bacterial probably accounts for the low values observed in pooled stored urine samples. Since, in contrast with the human (Corcoran *et al.*, 1951; Dustan, Mason and Corcoran, 1953), the yield of FC in rats was not increased by enzymatic hydrolysis, this procedure was not made part of this study.

RESULTS

I. Normal Rats: Oral Administration

Results are summarized in Table 1. Stated control mean value of FC is that described above (11.2 μg.) since determinations in each group proved unnecessarily tedious. Stated mean control levels of 17-KS are those found in each group. Values of each during treatment are noted for each day and also as the mean of the treatment period. The tabulated post-treatment value is derived from analyses of urine collected during the first post-treatment day. To facilitate estimates of the effects of treatment the mean treatment levels (A) are compared with mean control levels (B) and the ratio A/B presented in the table.

By compounds, the following effects were observed:

(a) desoxycorticosterone acetate: increase in FC and no change in mean 17-KS during treatment but an increase thereafter; in terms of the injected steroid, the yield of FC is 0.11%.

(b) cortisone acetate: increases in FC and 17-KS during treatment; FC yield is 0.63%.

(c) hydrocortisone acetate: increases in FC and 17-KS roughly as observed with cortisone acetate; FC yield 0.8%.

(d) cortisone: increases in FC and 17-KS; FC yield 1.07%.

(e) hydrocortisone: increases in FC and 17-KS; FC yield 0.8%. Yields of 17-KS from hydrocortisone and cortisone alcohols are somewhat less than were observed during treatment with their respective acetates.

(f) dehydrocorticosterone and corticosterone acetates do not increase urinary 17-KS; FC was not observed.

(g) testosterone: no change in FC; increase in 17-KS with a net yield of 11% as 17-KS.

The outputs during the first post-treatment days show abrupt remissions of the changes observed during treatment to values approximating or less than those of control periods.

II. Normal Rats: Subcutaneous Administration

Data are summarized in Table 2 in a manner corresponding to that of Table 1. Only 3 compounds were tested.

(a) desoxycorticosterone acetate: in contrast to oral administration, there is no change in FC; changes in 17-KS correspond to those described above.

(b) cortisone acetate: treatment increases FC and 17-KS as during oral administration.

(c) hydrocortisone acetate: increases in FC and 17-KS during treatment; net yield of FC is only 0.19%, which is one-quarter of the yield found after oral dosage; net increase of 17-KS was one-third less than was found after oral dosage.

In contrast with oral dosage, the values found on the first post-treatment day are has high as those found during treatment; decreases to or towards normal values occur during the 3 succeeding days.

III. Subtotally Hepatectomized Rats

Data are summarized in Table 3 in the manner of Table 1.

(1) Effect of the operation: FC increases abruptly and then decreases to subnormal values over the 5 postoperative days; the mean change is an increase. Roughly parallel changes occur in 17-KS, although the mean shows no change.

(2) Effects of steroids: These, with the exception of desoxycorticosterone, were given only orally. In the ratios A/B of FC, net A (treatment mean) is calculated as observed value less the mean postoperative value (25 μg.) in the group. The ratios of 17-KS are not similarly "corrected," because of the rough equality of pre- and postoperative means in the control group.

(a) desoxycorticosterone acetate: orally, FC increases and 17-KS is unchanged, as in normal animals; subcutaneously, it slightly increases FC.

(b) cortisone acetate: the increase in FC is more than twice that found in normal animals, while the increase in 17-KS is the same.

(c) hydrocortisone acetate: again, the increase in FC is roughly twice that found in normal animals; the output of extra 17-KS was not affected by the operation.

(d) cortisone: the increase in FC is roughly 4 times that found in normal animals; 17-KS excretion is not affected.

(e) hydrocortisone: the increase in FC is 3 times that found in normal animals; again, 17-KS output is not altered.

(f) dehydrocorticosterone acetate: no values of FC output during treatment were available from normal animals; however, the augmentation of FC output is similar to that observed after cortisone and hydrocortisone; 17-KS output was slightly increased by this compound in contrast to the observations in normal animals reported above.

(g) corticosterone acetate: only 17-KS was observed; as with dehydrocorticosterone acetate, the output was slightly increased by steroid treatment.

In these experiments, as in those in normal animals given steroids orally, outputs fell to or towards control levels abruptly on cessation of treatment.

DISCUSSION

The mechanism of renal excretion of steroid hormones and their metabolites is obscure. Certain presumptions seem justified. Most circulating steroid hormone is protein-bound and not available for renal excretion by glomerular filtration. The fraction of steroid in free solution, and that conjugated in a relatively water-soluble form, are subject to glomerular filtration. Consequently, the determinant of the urinary output of administered steroids or of steroid metabolites is the concentration of free and combined steroid in plasma water. This function at constant dosage, rate of absorption and protein concentration, will vary inversely with the rate of steroid catabolism. The variables tested in the above experiment can be considered in terms of these considerations.

(a) The Nature of the Compound. Both cortisone and hydrocortisone, either as alcohol or acetate, without regard to the route of administration or the integrity of liver function, increase urinary 17-KS; in normal animals acetates of desoxycorticosterone, dehydrocorticosterone and corticosterone do not. This accords with previous demonstrations, cited above, that C-17-hydroxylated corticosteroids act as 17-KS precursors. In some observations in human beings cortisone has been observed to decrease urinary 17-KS, presumably by suppressing endogenous cortical secretion (Polley and Mason, 1950; Villa, Ballabio and Sala, 1952). However, in our experiments and in clinical observations of increased post-cortisone 17-KS outputs, the doses used have been large.

All corticosteroids tested increased urinary FC. The largest yields followed administration of cortisone and the least that of desoxycorticosterone acetate. Presumably this reflects the more rapid absorption of cortisone and its comparatively large solubility in plasma (Macek, *et al.*, 1952).

The independent variations of FC and 17-KS seen in disease can, from the above observations, be attributed to changes in the composition of adrenal cortical secretion; this secretion is believed normally to contain largely hydrocortisone, a 17-hydroxylated steroid, and corticosterone (compound B) which has no 17-hydroxyl (Reich, Nelson and Zaffaroni, 1950). Changes in the relative proportions of these two constituents would account for the variable relationship of urinary 17-KS and FC; thus, an increase in the corticosterone content would increase FC while 17-KS would be unchanged or decreased. In this connection also, unstable and frequently high outputs of FC are found in many patients with hypertensive disease (Corcoran, Page and Dustan, 1950); the low or absent yields of FC observed during treatment of rats with desoxycorticosterone indicate that these increases in FC output cannot be attributed to increased secretion of desoxycorticosterone-like compounds. Rather, as noted elsewhere (Dustan, Mason and Corcoran, 1953) the increments in pH 1 extractable FC found in hypertension can be ascribed to decreases in the proportion of FC excreted as glucuronide.

(b) The State of Esterification. The comparisons here available are between alcohols and acetates of cortisone and hydrocortisone. Orally, the alcohols yielded somewhat less 17-KS than the acetates in normal rats; outputs of FC were roughly the same with both forms of the steroids. In human beings, however, the orally administered acetates seem more effective in increasing 17-KS than do the alcohols (Fajans, Louis and Conn, 1951 a).

(c) The Route of Administration. As concerns 17-KS, the notable difference between oral and subcutaneous administration is the greater increase elicited by oral hydrocortisone acetate. This difference is even greater in the case of FC; it prevails, in lesser degree, in the increment of FC which follows oral desoxycorticosterone acetate and the lack of effect on FC of this steroid given subcutaneously. Similarly, in human beings, oral hydrocortisone acetate causes greater increments in urinary 17-KS and FC than does intramuscular (Fajans, Louis and Conn, 1951 a). In this regard, Nelson *et al.*, (1952) found that oral dosage with either hydrocortisone or cortisone acetates causes a prompt rise in plasma 17-hydroxysteroid concentration, while intramusuclar dosage has little or no effect. This difference reflects slow absorption from intramuscular deposits dependent on the poor solubility of the steroid esters in body fluids (Macek *et al.*, 1952). This slow absorption has the effect that excretion of FC from subcutaneously deposited corticosteroid continues over several days, whereas the effect of oral corticosteroid on FC disappears on the day after withdrawing treatment.

(d) Effects of Subtotal Hepatectomy. Patients suffering from moderate or advanced hepatic cirrhosis sometimes show increases in urinary RC and FC associated with decreases in 17-KS (Bongiovanni and Eisenmenger, 1951); these abnormalities can be attributed to defective hepatic catabolism of normal or increased amounts of endogenous corticosteroids. On the other hand, normal values of urinary FC were observed by Mason (1951) in a group of jaundiced patients with various forms of liver disease; the same was observed (Butt *et al.*, 1951) in 2 jaundiced patients in whom also cortisone administration elicited roughly "normal" increases of urinary FC and actual decreases of 17-KS. More recently Chapman *et al.*, (1952) found that cortisone treatment did not alter 17-KS excretion in 3 cirrhotics. Thus, experiments in 2 jaundiced and 3 cirrhotic patients suggest that the low outputs of 17-KS in liver disease may be due to decreased hepatic synthesis of 17-KS from precursor steroids; on the other hand, the data of Mason (1951) and of Butt *et al.* (1951) do not agree with the concept that hepatic disease is associated with inadequate degradation of the corticosteroid side-chain. The above experiments in subtotally hepatectomized rats were designed to explore these mechanisms as affected by one type of hepatic insufficiency.

The operation itself caused a transient (3 days) increase in FC with somewhat parallel changes in 17-KS; both presumably reflect the release and conversion of endogenous corticosteroids as a result of trauma.

The effects of exogenous steroids on urinary FC and 17-KS in the presence of experimental hepatic insufficiency can best be observed by comparisons of the ratios A/B as between Tables 1 and 3.

TABLE 1. EFFECT OF ORALLY ADMINISTERED STEROIDS ON 17-KS AND FC IN NORMAL RATS

Outputs in μg. per 24 hours

Compound		Control (B)	Treatment days					Treatment mean (A)	Post-treatment 1st day	A/B
			1	2	3	4	5			
Desoxycorticosterone Acetate	FC	11.2	29	28	15	18	19	22	12	2.0
	17-KS	190	174	157	195	113	230	174	293	.9
Cortisone Acetate	FC	11.2	98	126	46	63	37	74	9	6.6
	17-KS	144	478	441	738	494	384	507	129	3.5
Hydrocortisone Acetate	FC	11.2	86	152	73	73	69	91	5	8.1
	17-KS	73	291	343	189	427	245	299	95	4.0
Cortisone	FC	11.2	66	—	107	108	192	118	13	10.5
	17-KS	112	172	235	417	—	146	242	79	2.2
Hydrocortisone	FC	11.2	120	56	96	92	—	91	7	8.1
	17-KS	114	263	196	192	264	461	275	219	2.4
Dehydrocorticosterone	17-KS	179	167	181	153	216	—	179	166	1.0
Corticosterone Acetate	17-KS	166	170	175	144	—	—	163	179	1.0
Testosterone	FC	11.2	20	13	9	11	15	14	7	1.3
	17-KS	189	949	475	1048	2380	1220	1214	218	6.4

TABLE 2. EFFECTS OF SUBCUTANEOUSLY ADMINISTERED STEROIDS ON 17-KS AND FC IN NORMAL RATS

Outputs in μg. per 24 hours

Compound		Control (B)	Treatment days					Treatment mean (A)	Post-treatment 1st day	A/B
			1	2	3	4	5			
Desoxycorticosterone Acetate	FC	11.2	21	14	14	5	6	12	N.D.*	1.1
	17-KS	199	182	151	256	180	220	197	304	1.0
Cortisone Acetate	FC	11.2	58	66	71	100	67	72	106	6.4
	17-KS	118	440	429	491	333	504	439	453	3.7
Hydrocortisone Acetate	FC	11.2	28	12	47	26	37	30	34	2.7
	17-KS	87	114	285	188	310	290	237	231	2.7

* N.D. = Not detectable.

First, subtotal hepatectomy does not alter new formation of 17-KS from exogenous corticosteroid. In this respect, this experimental insufficiency contrasts with that observed in the 2 patients of Butt *et al.*, (1951) whose 17-KS outputs decreased during treatment with cortisone. Evidently the

TABLE 3. EFFECTS OF ORALLY ADMINISTERED STEROIDS ON 17-KS AND FC IN RATS SUBTOTALLY HEPATECTOMIZED

Outputs in μg. per 24 hours

Compound		Control (B)	Treatment days					Treatment mean (A)	Post-treatment 1st day	A/B
			1	2	3	4	5			
———	FC	11.2	61	31	21	1	9	25	N.D.*	2.2
	17-KS	158	213	154	114	140	215	167	177	1.1
Desoxycorticosterone Acetate	FC	11.2	66	42	49	30	43	46	6	1.87
	FC†	11.2	64	46	36	49	50	49	21	2.14
	17-KS	164	174	132	136	190	299	182	344	1.1
Cortisone Acetate	FC	11.2	98	151	270	294	121	187	5	14.4
	17-KS	175	336	588	560	521	531	507	185	2.9
Hydrocortisone Acetate	FC	11.2	189	296	362	130	64	208	11	16.3
	17-KS	159	408	697	1151	621	555	686	134	4.3
Cortisone	FC	11.2	562	550	500	274	—	471	16	39.8
	17-KS	124	341	455	264	196	257	300	132	2.4
Hydrocortisone	FC	11.2	484	296	292	362	125	312	10	25.6
	17-KS	176	660	599	558	429	422	534	165	3.
Dehydrocorticosterone Acetate	FC	11.2	312	349	300	292	—	313	N.D.*	25.7
	17-KS	134	370	252	182	155	—	239	133	1.8
Corticosterone Acetate	17-KS	172	277	364	316	378	—	334	146	1.9

* N.D. = Not detectable.
† Subcutaneous Administration.

kind or degree of hepatic insufficiency elicited by sub-total hepatectomy is not enough to alter the conversion of corticosteroid to 17-KS; from this it would seem likely that deficient hepatic formation of 17-KS is not an adequate explanation of the low 17-KS outputs seen in chronic liver disease. Rather, the defect may be akin to that which decreases 17-KS output in most debilitating illness.

Secondly, the data show that subtotal hepatectomy increases FC output in response to injections of corticosteroids other than desoxycorticosterone. Since degradation of the steroid side-chain is effected in part by the liver (Schneider and Horstman, 1951; Louchart and Jailer, 1951), the effect of sub-total hepatectomy on FC output can be attributed to defective hepatic degradation of the steroid side-chain. In this respect also, the subtotally hepatectomized rat differs from the 2 patients observed by Butt *et al.* (1951). The data accord with those of Bongiovanni and Eisenmenger (1951), whose patients were observed during illness due to cirrhosis and ascites; the disparate observations of Mason (1950) were made in patients observed because of the presence of jaundice.

SUMMARY AND CONCLUSIONS

Observations of urinary outputs of 17-KS and FC were made in normal and subtotally hepatectomized rats given exogenous corticosteroids in large doses over brief periods.

The data confirm the function of C-17-hydroxylated corticosteroids as 17-KS precursors. The variable excretions of 17-KS and FC in clinical disease can be attributed to variations in the content of C-17-hydroxylated steroid in the secretion of the adrenal cortex.

Among the compounds tested, cortisone alcohol caused the greatest and desoxycorticosterone acetate the least increase in FC. As between the steroid alcohols and acetates, both yielded comparable increments of FC; the alcohols yielded somewhat less17-KS.

Oral, as compared with subcutaneous administration was more effective in increasing urinary FC; this difference was especially marked with hydrocortisone and desoxycorticosterone.

Provisions of exogenous corticosteroids to subtotally hepatectomized rats augment the output of FC, presumably because of impaired hepatic degradation of the steroid side chain; the new formation of 17-KS from exogenous steroid is not impaired. Consequently, while the increase of FC sometimes seen in chronic liver disease can be attributed to defective liver function, the decreases of 17-KS which commonly occur in such conditions may reflect extrahepatic effects of chronic disease.

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THE LAURENTIAN HORMONE CONFERENCE

The Laurential Hormone Conference of the AAAS will hold its 1953 annual meeting at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 6–11. Interested investigators and specialists in the hormone field may apply for attendance by writing to the Committee on Arrangements, 222 Maple Avenue, Shrewsbury, Mass., for application blanks. Since accommodations at the hotel necessarily limit the attendance, only those persons submitting applications can be considered. The application blanks must be received by the Committee no later than *May 15* in order to insure issuance of invitations as soon as possible thereafter.

The following program has been arranged:

I. *Nervous system-hormone interrelationships*
  The central nervous system and stress-induced eosinopenia.
    Dr. R. W. Porter, Univ. of Calif. School of Medicine at Los Angeles
  Studies of brain metabolism and electrical activity in relation to adrenocortical physiology.
    Dr. Hudson Hoagland, The Worcester Foundation for Experimental Biology
  Effects of hormones on cerebral function.
    Dr. D. M. Woodbury, Univ. of Utah School of Medicine
II. *Thyroid Hormone physiology and biochemistry*
  Triiodothyronine in relation to thyroid physiology.
    Drs. J. Gross and R. Pitt-Rivers, National Inst. for Medical Research, London, and State Univ. of New York Medical Center
  Metabolic effects of thyroid hormones *in vitro*.
    Drs. H. A. Lardy and G. Feldott, Univ. of Wisconsin School of Medicine
III. *Comparative endocrinology*
  Endocrine mechanisms in the life of insects.
    Dr. D. Bodenstein, U. S. Army Chemical Center, Maryland
  Hormones produced by neurosecretory cells.
    Drs. Ernst and Berta Scharrer, Univ. of Colorado Medical Center
IV. *Protein Hormones*
  The preparation and chemistry of crystalline insulin.
    Dr. R. G. Romans, Connaught Medical Research Laboratory
  The chemistry of ACTH.
    Drs. E. E. Hays and W. F. White, The Armour Research Laboratories
V. *The role of hormones in blood and blood-forming organs*
  Endocrine factors and radiation-induced lymphoid tumors of mice.
    Dr. Henry S. Kaplan, Stanford Univ. School of Medicine
  Endocrine influences upon the formed elements of blood and blood-forming organs.
    Dr. Albert S. Gordon, Washington Square College, New York Univ.
VI. *Aspects of Clinical Endocrinology*
  Some problems related to ovarian function and to pregnancy.
    Dr. B. Zondek, Rothschild Hadassah Univ. Hospital, Jerusalem
  Clinical studies on electrolyte and fluid metabolism.
    Dr. Rolf Luft, Serafimerlasarettet, Stockholm
  Endocrine-metabolic studies in man.
    Dr. J. W. Conn, Univ. of Michigan School of Medicine

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THYROTROPIN ASSAY BASED ON THE DEPLETION OF IODINE IN CHICK THYROIDS

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THYROTROPIN assays have been based on either the secondary effects due to the increased activity of the stimulated thyroids or on one or more of the specific effects induced on the thyroid gland by the TSH stimulation of the glandular secretory mechanism. The former assays have been predominantly based on the accelerated metamorphosis of the amphibian larvae (Cuyler *et al.*, 1936; D'Angelo and Gordon, 1950), while the latter assays have been based on the increase in thyroid weight (Rowlands and Parkes, 1934; Smelser, 1938; Bergman and Turner, 1939), on the increase in acinar cell height (Starr and Rawson, 1937; Rawson and Salter, 1940; Adams, 1942), on the appearance of a histological response (Jorgensen and Wade, 1941; Ciereszko, 1945), on the increase in the intracellular colloid droplets of the thyroid gland (Dvoskin, 1947) and on the decrease in the thyroid iodine content (Stimmel *et al.*, 1936; Cuyler *et al.*, 1936). Excellent reviews of the existing thyrotropin assay methods have been included in the articles by White (1944), Adams and Beeman (1946) and Albert (1949).

The method presented in this paper is based on the depletion of iodine in the thyroids of chicks. It is dependent on the comparison of the responses obtained from TSH preparations of unknown potencies with the responses elicited by 2–3 levels of a standard TSH preparation run concomitantly.

MATERIALS AND METHODS

White Leghorn cockerels (from several midwest hatcheries) 1–2 days of age, averaging 30–45 gm. in body weight were utilized. These chicks, kept in a brooder at a fairly

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[1] Deceased.

constant temperature, were maintained on a commercial chick starter mash (Purina).

Three different kinds of materials were used as reference TSH standards at the three stages of assay development. Initially, beef anterior pituitary acetone powder, BAP #1, was used. This was then superceded by 2R3 with an assumed TSH potency of 100%. It was a partially purified TSH obtained by alkaline-alcohol fractionation of a beef anterior pituitary acetone powder. Its hormonal contaminants included ACTH (.06 I.U. per mg. by the ascorbic acid depletion method), gonadotropin (approximately 6 Collip units per mg.), growth hormone (approximately 15% of pure beef growth hormone by the 10 day weight gain method in hypophysectomized rats), oxytocin (.03 U.S.P. units per mg.) and pressor (less than 0.03 U.S.P. units per mg.). Finally the U.S.P. Reference Thyrotropin replaced 2R3 as the standard. One U.S.P. thyrotropin unit is that amount of thyrotropin activity contained in 20 mg. of the U.S.P. Reference Substance; in terms of activity, 2.70 mg. of 2R3 is equivalent to one U.S.P. unit.

The samples containing unknown amounts of thyrotropin included 317-51 which was used in the preparation of the U.S.P. Thyrotropin Reference Substance, various TSH fractions, clinical FSH, ACTH, LH and growth fractions as indicated below.

Upon arrival, 100–200 chicks were individually marked with colored dyes, randomly distributed into ten groups of approximately equal size and injected subcutaneously with 0.2 ml. of an appropriate solution. The groups consisted of a control group injected with saline, 2–3 groups treated with 2–3 levels of the standard TSH BAP #1 (2R3 or U.S.P. Thyrotropin Reference Substance) and several groups treated with thyrotropin fractions of unknown potencies (1–2 levels each). The TSH samples were dissolved in saline at approximately pH 7.5 with the aid of 0.1 N NaOH and stored in a deep freeze between injections.

The chicks were injected once daily for three days. On the fourth day, food was withheld very early in the morning. Twenty-four hours after the last injection, the chicks were killed with ether. The thyroids were carefully dissected out, as described by Cope (1938), rapidly weighed to the nearest 0.1 mg. on a Roller-Smith torsion balance and placed in vials containing redistilled 95% ethanol. One to two pairs of these thyroids were stored in each vial so that each group had ten vials for ten iodine determinations. The thyroids were then assayed for the total iodine content according to the method of Walaszek-Piotrowski and Koch (1952), the results being calculated as micrograms of iodine per milligram of wet thyroid tissue.

The iodine responses elicited in the thyroids of the chicks injected with the TSH samples of unknown potencies were compared with the responses of the chicks treated with the reference TSH. Potency ratios and standard errors were calculated by means of the Irwin log dose method (1937). The potencies were reported as percentages of TSH activity of standard 2R3 or as U.S.P. units of TSH per mg. sample.

RESULTS

As the initial source of thyrotropin, beef anterior pituitary acetone powder, BAP #1, was utilized. For injection purposes, it was triturated with saline, brought up to a volume containing 50 mg. of total solids per ml., extracted at a pH of 7.5 for four hours with constant stirring at room temperature and centrifuged. The resultant supernatant containing 30 mg. of total protein per ml. was then used as a reference standard for the assay of TSH. The doses 1.1, 2.2 and 4.5 mg. of BAP #1 gave a graduated decrease of thyroid iodine such that, when the responses (μg. iodine per mg. thyroid) were plotted against the log doses, a straight line resulted. When the dosage of this pituitary standard TSH was extended to include up to 36 mg. of BAP #1, the iodine responses tended to plateau while the thyroid weight responses did not (see Fig. 1). Consequently, 1.1, 2.2 and 4.5 mg. of BAP #1 were used as a standard reference dose, and the thyroid iodine responses were used as the basis for TSH assay calculation.

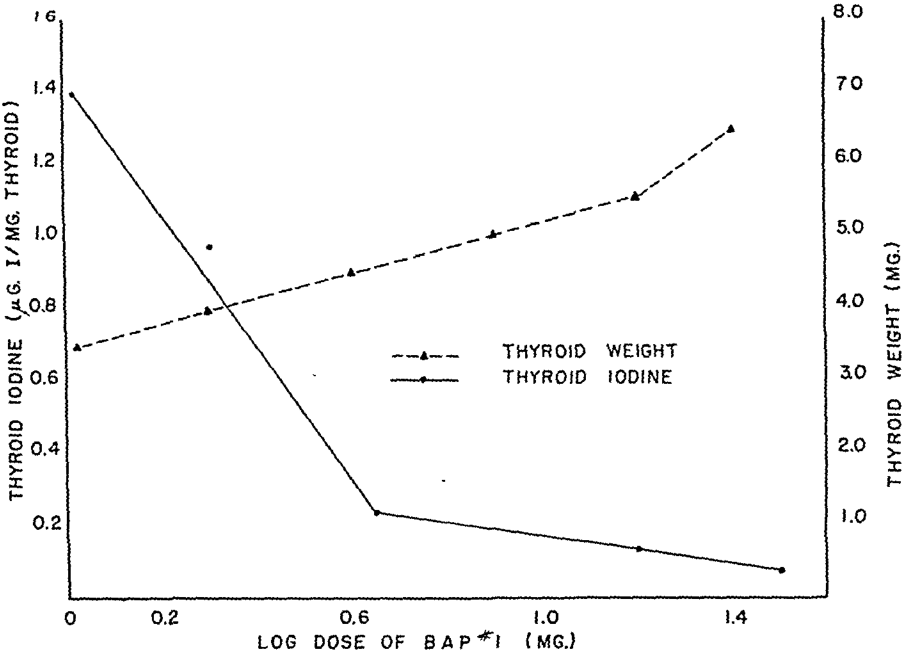

FIG. 1. Depletion of thyroidal iodine and increase in thyroid weight with acetone powder of beef anterior pituitary

As the fractionation of thyrotropin from beef anterior pituitary acetone powder proceeded, a large quantity of purer TSH fraction, 2R3, was obtained. It assayed 2100±600% of BAP #1 and since it was only slightly contaminated with other pituitary hormones, it replaced BAP #1 as the reference standard. A total dose of 80 μg. of this TSH fraction gave a 30–50% decrease in thyroid iodine usually without any thyroid weight response. The total doses finally utilized were 25 μg., 50 μg., and 100 μg. or 40 μg., 80 μg., and 150 μg. A typical assay is shown in Table 1. The samples tested were various thyrotropin preparations with the exception of K4540SR, a clinical FSH sample.

As a final step in this assay development, a U.S.P. Thyrotropin Reference Substance replaced the 2R3 as the reference standard. For purposes

TABLE 1. EXPERIMENTAL TSH ASSAY NO. 165

Control	Standard 2R3			TSH #3	TSH 2+3RW	K45408-R	317-51	U.S.P. TSH	
	0.040 mg.	0.080 mg.	0.160 mg.	0.080 mg.	0.080 mg.	0.530 mg.	0.032 mg.	0.016 u.	0.032 u.
Average Thyroid Weight (mg.)									
3.2_{15*}	3.2_{14*}	3.2_{15*}	3.7_{15*}	3.4_{15*}	3.7_{13*}	3.8_{15*}	3.2_{15*}	3.5_{15*}	3.5_{15*}
Thyroid Iodine Content (µg. I/mg. thyroid)									
1.64	1.43	1.12	0.58	1.28	0.95	0.63	1.01	1.39	0.90
1.33	1.38	0.88	0.44	1.03	0.76	0.52	0.98	1.18	0.81
1.21	1.17	0.81	0.44	0.82	0.66	0.44	0.93	1.16	0.66
1.20	0.92	0.73	0.42	0.71	0.60	0.38	0.57	0.97	0.61
0.97	0.83	0.68	0.41	0.63	0.41	0.35	0.54	0.91	0.60
0.91	0.80	0.57	0.34	0.61	0.40	0.33	0.51	0.69	0.42
0.88	0.74	0.49	0.32	0.57	0.39	0.32	0.48	0.54	0.41
0.81	0.73	0.44	0.31	0.54	0.35	0.22	0.40	0.41	0.28
0.77	0.68	0.39	0.17	0.40	0.32	0.21	0.37		0.27
0.70	0.60	0.23		0.36	0.25	0.15	0.34		
1.04	0.93	0.63	0.38	0.70	0.51	0.36	0.61	0.91	0.55

* Subscripts indicate number of animals per group.

Using Irwin's log dose method for calculation of TSH potencies, the results are:

TSH #3	88 ± 20%	of Standard TSH 2R3
TSH 2+3RW	140 ± 33%	of Standard TSH 2R3
K45408-R	31 ± 8%	of Standard TSH 2R3
317-51	280 ± 65%	of Standard TSH 2R3
USP	300 ± 55%	of Standard TSH 2R3

of assay, the doses 0.0125, 0.025, and 0.050 units of U.S.P. TSH have been used (these are approximately equivalent to 40 µg., 80 µg., and 160 µg. of thyrotropin sample 2R3). The 0.025 unit dose decreased the iodine contant of the chick thyroids 30–60% without usually affecting the thyroid weight.

DISCUSSION

Cockerels other than the White Leghorns used were tested for their sensitivity to TSH but were found to be unsuitable. The normal guinea pig and hypophysectomized rat were found to be very much inferior to the two day old White Leghorn cockerel. The guinea pig was found to be especially insensitive to TSH as 2.0 mg. of TSH #2R3 gave no decrease in thyroid iodine and only 35% increase in the mean thyroid weight. On the other hand, a dose of 2.0 mg. TSH #2R3 in the hypophysectomized rat caused a mean 18% decrease in thyroid iodine as compared to hypophysectomized controls and no increase in thyroid weight.

The White Leghorn cockereles have been found, thus far, to be the only breed of chicks very sensitive to thyrotropin. However, there were periods of variation in their sensitivity to TSH. These chicks seemed to be most sensitive in June and August, and least in April, November and December. To avoid variations in responses, a routine of injecting twice daily for three days was studied, but this resulted in responses no different than the one injection per day routine. This could be explained readily because of the prolonged action of TSH on the thyroid (see Table 2), the effect being apparent 72 hours after a single injection of TSH. A one dose assay was then studied. One hundred chicks were injected once subcutaneously with 0.2 ml. solution, autopsied two days later, and the thyroid iodine determinations were performed as described above. The responses were poor. There was a 12% decrease in iodine at the 12 μg. level of 2R3, a 40% decrease at the 50 μg. level and only a 54% decrease at the 3.2 mg. level. Finally the thyroid weight response method was investigated and resulted in poorly graded, variable responses to graded TSH doses (0.4 gm. to 3.2 mg.). Consequently, the TSH preparations, in this laboratory, have been assayed using the iodine depletion of the thyroids of two day old White Leghorn cockerels as described above.

TABLE 2. THYROID IODINE OF CHICKS INJECTED ONCE WITH 3 MG. OF 2R3 AND AUTOPSIED AT VARIOUS INTERVALS

Hours after injection	Number of chicks	Average thyroid weight, mg.	Average* thyroid iodine, γ/mg.
0	10	3.6	2.26
0.5	10	3.4	2.39
1	10	3.8	2.25
2	10	3.5	1.96
3	10	4.7	1.71
4	10	4.3	1.65
7	10	4.3	1.59
24	10	3.4	1.46
32	10	3.3	1.41
48	10	3.4	1.27
72	10	4.2	1.31
72 (Control)	10	3.9	2.20

* One determination on the pool of all the thyroids of one group.

To note whether the thyrotropin potencies as obtained by this method were duplicable, two samples of TSH #317-51 and TSH #3 were subjected to several TSH assays over a period of several months using the chicks from one source (Berry's Hatchery, Illinois). These two TSH fractions were of different degrees of purity and activity; TSH #3 was a by-product of TSH fractionation while #317-51 was used in the preparation of the U.S.P. Thyrotropin Reference Substance. The results are shown in Table 3. The weighted mean standard errors obtained were 14.5% for TSH #3 and 7% for 317-51.

To note if the TSH contamination of other pituitary products could be detected by this method, a series of crude and potent hormonal fractions (other than TSH) were assayed. The results obtained (see Table 4) indicated that this assay method was very sensitive and could detect TSH contamination in anterior pituitary hormonal fractions at levels as low as 0.3% of 2R3.

TABLE 3. THYROTROPIN ASSAY RESULTS ON TSH #3 AND 317–51

Sample	Assay No.	Reference standard	Levels of sample per assay	TSH potency	
				% of 2R3	U.S.P. u./mg.*
TSH #3	160	2R3	1	170 ± 78	
	161	2R3	1	70 ± 34	
	162	2R3	1	93 ± 43	
	165	2R3	1	88 ± 20	
	166	2R3	1	103 ± 26	
Weighted Mean				89 ± 13	
317–51	149	2R3	2	270 ± 78	1.0 ± 0.3
	150	2R3	2	350 ± 140	1.3 ± 0.5
	153	2R3	1	180 ± 30	0.7 ± 0.1
	154	2R3	3	220 ± 28	0.8 ± 0.1
	165	2R3	1	280 ± 65	1.2 ± 0.3
	166	2R3	2	250 ± 43	1.0 ± 0.2
	167	2R3	1	340 ± 65	1.4 ± 0.3
	168	USP	1		0.6 ± 0.2
	171	USP	2		1.3 ± 0.4
	172	USP	2		0.9 ± 0.3
	173	USP	2		1.6 ± 0.2
	174	USP	2		1.4 ± 0.2
Weighted Mean				242 ± 17	1.1 ± 0.06

* 2R3 contains 0.37 U.S.P. units per milligram.

TABLE 4. TSH CONTENT OF SOME PITUITARY SUBSTANCES

Sample	% of standard TSH 2R3
Fresh beef anterior pituitary	0.8 ± 0.1
Beef anterior pituitary, acetone powder	5.0 ± 0.2
Sheep pituitary, acetone powder	1.7 ± 1.0
Fresh hog pituitary	1.0 ± 0.4
Hypothalamic extract	0
Prolactin, 2R-CR[1]	0
LH, FW-234[2]	9.7 ± 3.3
FSH, PFC-7C[3]	35.0 ± 11
Growth hormone 22KR2[4]	3.4 ± 3.1
ACTH 58-9(50)[5]	0.3 ± 0.1

[1] Contained 29 ± 8 International units of prolactin per mg.
[2] Assayed 113 ± 19 Collip units per mg.
[3] Assayed approximately 250 Collip units per mg.
[4] Armour's growth hormone standard having a potency of 100%.
[5] Assayed 1.8 International units ACTH per mg.

SUMMARY

A thyrotropin assay method based on the depletion of thyroid iodine has been developed which is dependent on a comparison (by means of the log dose method of Irwin) of responses obtained from unknown TSH preparations run at one or two levels to the responses obtained from two to three levels of a standard thyrotropin preparation. The two level assays gave TSH potencies with a standard error of approximately 25%.

Acknowledgment

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CORRELATION OF PREPARATIVE HISTORY AND METHOD OF ASSAY OF CORTICOTROPIN WITH CLINICAL POTENCY

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INTRODUCTION

IT IS well known that different ACTH assay methods give different results, but the reasons for this have not been fully elucidated.

Li (1952) has pointed out the discrepancies in evaluation of ACTH activity by different assay methods, which include the adrenal ascorbic acid depletion test of Sayers *et al.* (1948), the adrenal weight maintenance, and the hypertrophy and repair tests. Reinhardt *et al.* (1951) have emphasized the importance of applying several assay methods in the study of various ACTH preparations and the importance of absorption rates in assay methods. Discrepancies in potency estimates by the various assay means have also been noted by Young *et al.* (1951), Dixon *et al.* (1951) and Moyer *et al.* (1952). From the evidence presented there has frequently risen the postulate of two factors, one of which effects adrenal weight, and the second depletion of adrenal ascorbic acid.

Bruce, Parkes and Perry (1952) have studied the thymus involution response of nestling rats to ACTH and elaborated an assay method based upon this criterion of activity. Their method utilized litter-mate nestling rats, three daily injections of ACTH suspended in an oil-beeswax medium, and autopsy to obtain thymus weight twenty-four hours after the last injection.

In comparing the results of assay methods involving thymus or adrenal weight with the results obtained by the Sayers technique it must be borne in mind that the Sayers method utilizes intravenous injection of test doses while most other methods utilize subcutaneous or intramuscular injection.

There has been little clarification of the relative importance of routes of administration when different results have been obtained by different methods of assay. In addition, the variable results obtained by the several methods of assay have not been correlated with clinical activity. An abstract of the preliminary phase of these studies was reported by Fisher and Thompson (1952).

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MATERIALS AND METHODS

An experimental, purified ACTH in 16% gelatin standard was prepared for subcutaneous assay purposes. The term "purified" denotes preparations adsorbed and eluted from cellulose by methods originating in our laboratory and by the method described by Astwood *et al.* (1951). This standard was prepared by mixing equal unitage from each of three lots of purified ACTH in gelatin. These lots had been clinically assayed by several investigators and each was found to be two to four times as potent in terms of intravenously assayed International Units as other ACTH preparations in gelatin when administered subcutaneously or intramuscularly. On the basis of the assays on each lot by the intravenous method, and on the basis of intravenous assay of the finished mixture, the experimental standard contained 40 ± 4.0 I.U./cc. The standard has been stored frozen in small aliquots with no indication of potency change over a period of 12 months.

All test solutions contained the required dose of ACTH in 16% partially hydrolyzed U.S.P. gelatin (gel point about 23° C.) containing 0.5% phenol. All test samples in a given experiment were diluted with the same lot of gelatin.

The ACTH preparations used for all experiments reported (except when specified) in this study were derived from hog pituitary glands and were assayed in terms of I.U. to a standard error of 10% or less by the method of Munson *et al.* (1948) using hypophysectomized rats. All doses in subcutaneous experiments were given in terms of I.U.

The ACTH preparations referred to as crude have activities of less than 2 I.U./mg. as contrasted to the purified preparations, isolated by several different adsorption procedures, with activities between 20–60 I.U./mg. Preparations referred to as Type I forms are those which have been subjected to limited hydrolytic conditions during the process of isolation and the Type II and III forms are those subjected to more rigorous hydrolytic treatment, pepsin and pepsin plus acid, respectively. Types II and III are differentiated from Type IV in that the latter form has not been exposed to hydrolytic conditions.

The test animal in all cases was the Sprague-Dawley strain of rat. Intact and hypophysectomized animals of various ages were used as specified within each experimental design.

When adrenal ascorbic acid depletion was utilized as the end response following subcutaneous injection, individual groups of animals were sacrificed at suitable intervals after injection to obtain either a time response pattern or response at a specified time after administration of the hormone.

Preliminary experiments indicated that ACTH preparations prepared by different procedures were different in relative potency when assayed by the intravenous technique as compared to the subcutaneous technique employing either an aqueous or gelatin medium. Utilizing a gelatin medium, a greater response was obtained with relatively slight effect on the time pattern. A gelatin medium was employed throughout the following experiments because of the magnification of effect which could be obtained and because standardization of ACTH in gelatin was a primary problem.

All adrenal weight and thymus weight experiments reported in the present study involved the injection of 0.5 cc. test solution twice a day (9:00 A.M. and 4:00 P.M.) for three days, and autopsy the forenoon of the fourth day.

RESULTS

Figure 1 illustrates the results of a comparison of the adrenal ascorbic acid depleting activity of crude Type IV and crude Type I ACTH in saline, and in gelatin, after subcutaneous injection of 1.0 I.U./rat. Each point on the graph represents the average response of four rats. It may be seen that a gelatin medium produces a marked increase on the response to both types of preparations. However, the effect on the response to Type I ACTH appears considerably greater.

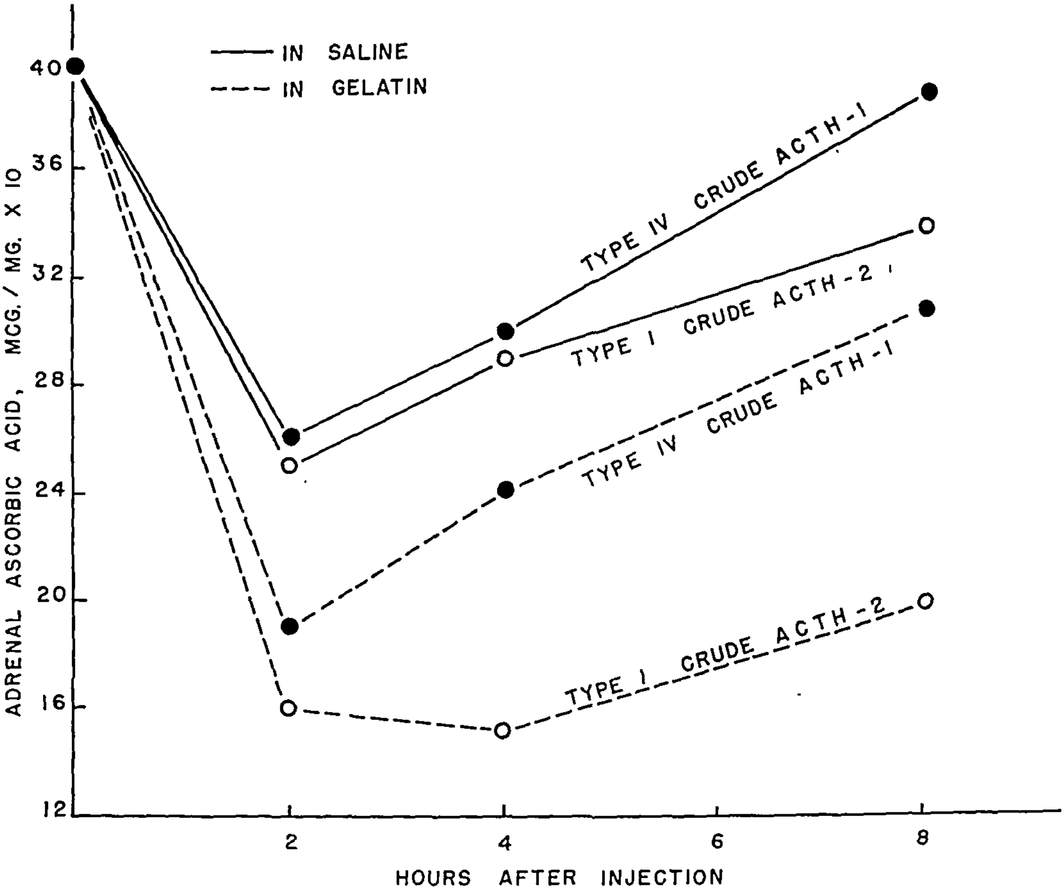

FIG. 1. Adrenal ascorbic acid response versus time after subcutaneous injection of two ACTH preparations in aqueous saline or gelatin vehicle. Each rat received 1.0 I.U. in 0.5 cc. Each point is the mean response value from four hypophysectomized rats.

In order to determine the relative importance of "purity" as compared with "preparative history" in obtaining higher subcutaneous potency in a gelatin medium, the method was converted to a quantitative design and several suitable ACTH samples were assayed in comparison with each other. Figure 2 illustrates graphically the results of such an experiment. Rats were sacrificed at 2 and 4 hours after injection for determination of adrenal ascorbic acid. Since there was no significant difference between the 2 and 4-hour response, these values were combined to produce the results illustrated in Figure 2. Each point then represents the average response of 8 rats.

In Figure 2, the ACTH sample identified as "Purified ACTH-247 (Type I)" assayed 30 I.U./mg., and the sample identified as "Crude ACTH (Type IV)" assayed 1.7 I.U./mg. When these samples in 16% gelatin medium were assayed clinically by intramuscular injection (Wolfson, 1952) the "Purified ACTH-247 (Type I)" was found to be two to four times as potent as the "Crude ACTH (Type IV)." It appears that there is good correlation between this assay method and clinical effectiveness. The sample identified as "Crude (Type I) ACTH-247" is the relatively crude extract (1.0 I.U./mg.) from which "Purified ACTH-247 (Type I)" was derived by selective adsorption. The sample "Purified

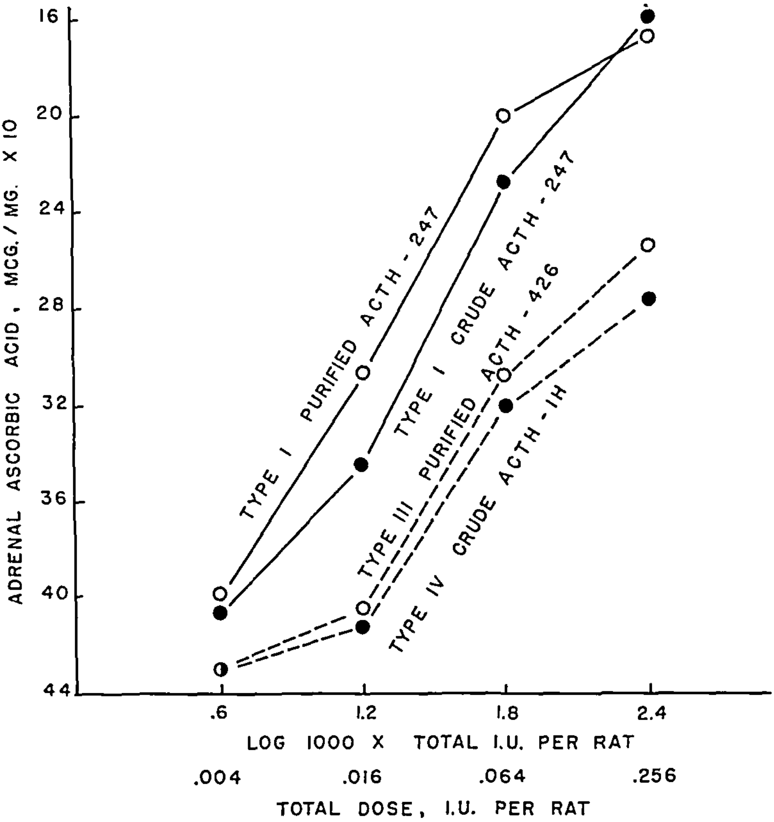

FIG. 2. Assay of several ACTH preparations subcutaneously administered and measuring adrenal ascorbic acid depletion in hypophysectomized rats—eight rats per point—three hour response.

ACTH-426 (Type III)" isolated by chromatopile partition (White *et al.*, 1951) assayed 50 I.U./mg.

In view of the apparent correlation between clinical assays (subcutaneous or intramuscular administration) and the potency value obtained utilizing the adrenal ascorbic acid depletion method (subcutaneous administration), it was anticipated that other subcutaneous methods utilizing normal rats and/or other criteria of activity such as adrenal weight or thymus weight might also correlate with clinical activity.

An experiment on 23 day old normal Sprague-Dawley rats, selected at

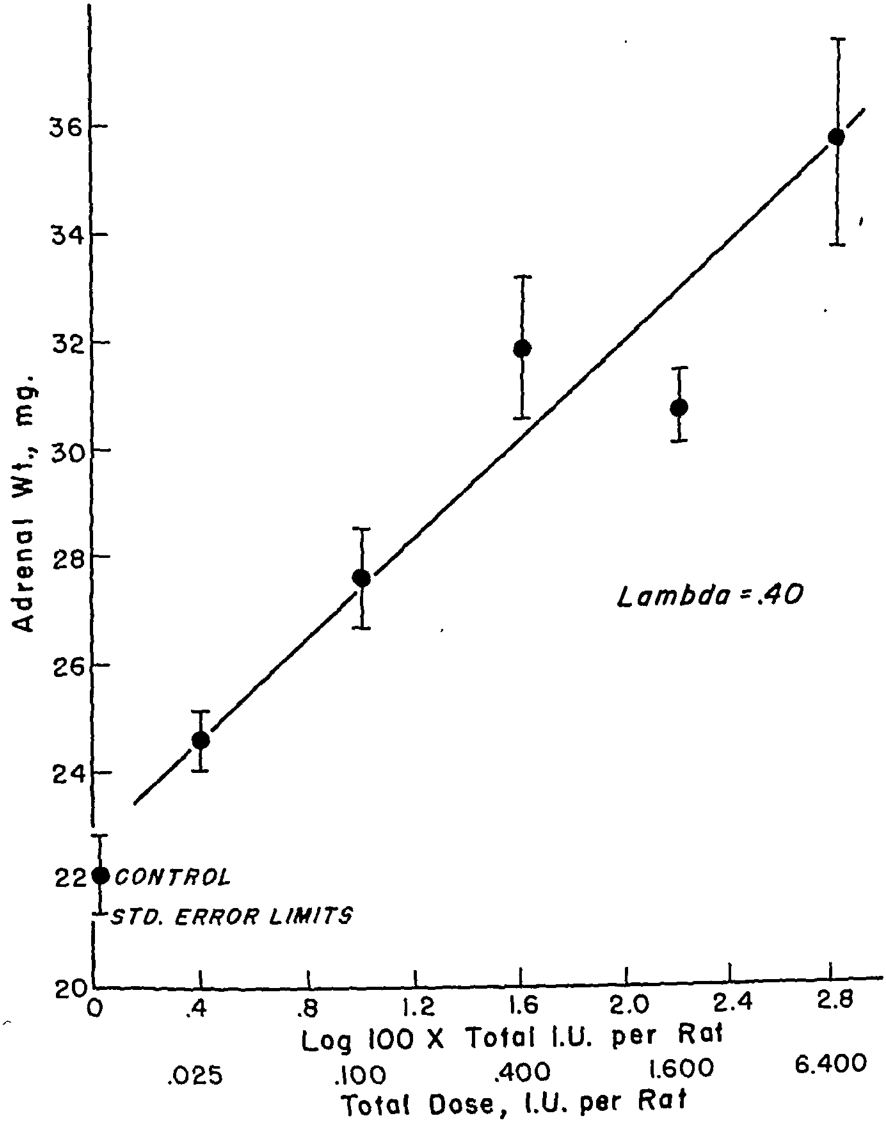

FIG. 3. Adrenal weight response of 23 day old, normal female rats to graded doses of purified ACTH in gelatin. 0.5 cc. subcutaneously twice a day for three days, autopsy fourth day, seven rats per point.

random, indicated that a useful adrenal weight response could be obtained on these animals but that the thymus involution response was more quantitative. Information from both responses could be obtained from the same rats (see also Hayashida and Li, 1952). One similar experiment on 30 day old rats indicated an adequate range of response using either adrenal weight or thymus weight but precision of the thymus response was poor in this experiment as compared to the 23 day old rats. Average control thymus weight was 323 mg. in this experiment and was reduced to 140 mg. with the highest dose of ACTH used (0.40 I.U. purified ACTH (Type I)—total dose in 3 days). Adrenal weight precision was comparable to results from 23 day old rats (lambda = .45).

Figure 3 illustrates the log-dose adrenal weight response relationship of purified ACTH (Type I) in gelatin administered as previously indicated to 23 day old normal female rats. The lambda value of 0.40 indicated relatively poor precision but is sufficiently valid to obtain information with

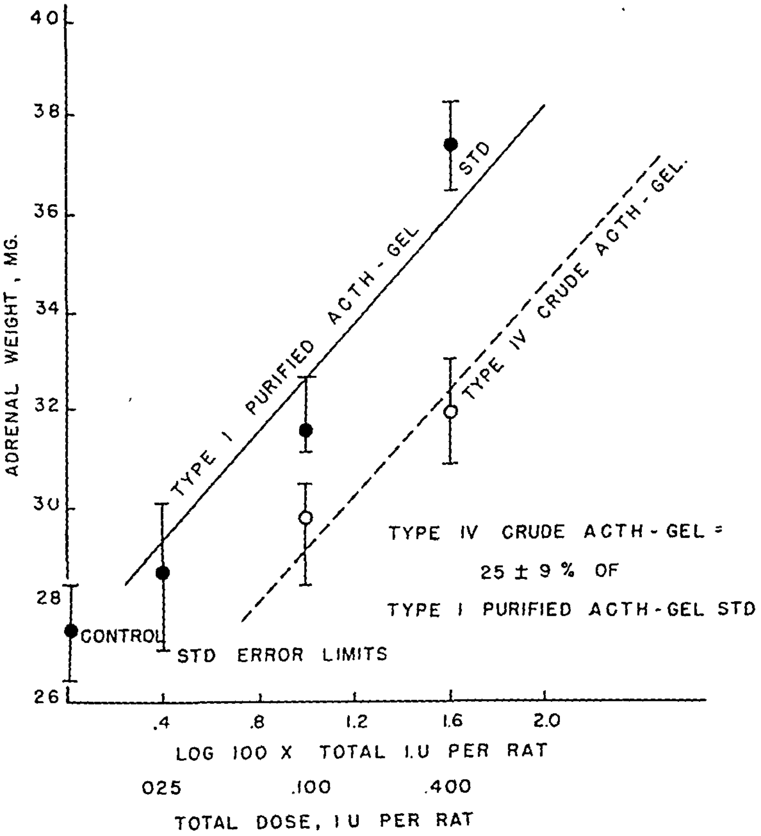

FIG. 4. Adrenal weight assay of ACTH. Thirty day old, normal, female rats—eight rats per point.

respect to correlation between adrenal weight assays and clinical or other assays.

Figure 4 illustrates the application of the adrenal weight response of normal 30 day old rats to the assay of crude ACTH (Type IV) in gelatin as compared to purified ACTH (Type I) in gelatin. The result—Type IV crude ACTH = 25 ± 9% of purified ACTH—is sufficiently accurate to indicate good correlation with clinical assay.

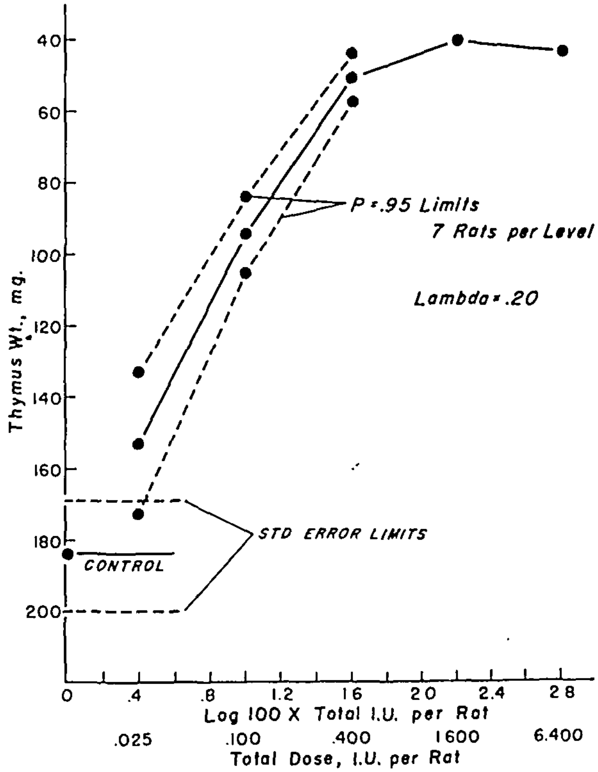

FIG. 5. Thymus weight response of 23 day old, normal, female rats to graded doses of purified ACTH in gelatin. This experiment also provided adrenal weight data shown in Figure 3.

Figure 5 illustrates the log-dose thymus weight relationship of purified ACTH (Type I) in gelatin administered to 23 day old female Sprague-Dawley rats selected at random. The thymus weights were obtained from the same rats that yielded the adrenal weight data illustrated in Figure 3. Thus it can be seen that in the dosage range of 0.10 to 0.40 I.U. of purified ACTH (Type I), quantitative data can be obtained simultaneously from both adrenal and thymus weight responses. In view of the correlation of standard deviation with level of response, conversion of the responses to log-response would seem indicated for assay purposes.

Figure 6 illustrates the assay curve obtained by using the logarithms of the thymus weight response values.

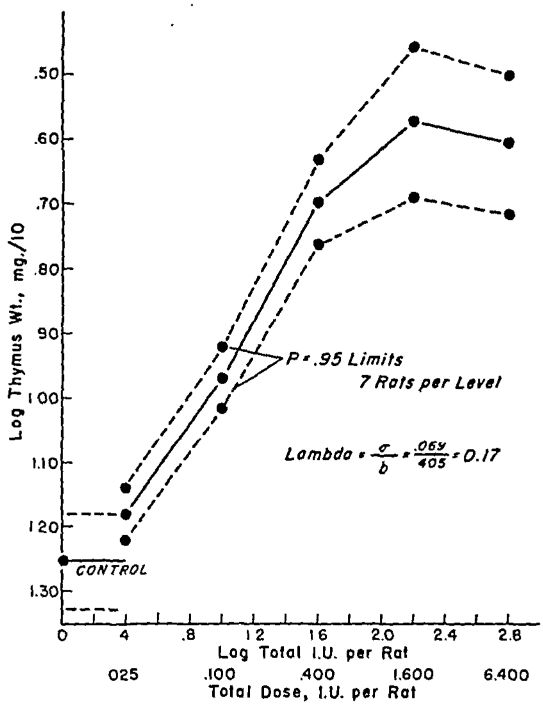

FIG. 6. Log thymus weight plotted against log dose of ACTH using data from experiment illustrated in Figure 5.

Figure 7 illustrates the application of the thymus involution assay procedure for the potency comparisons of several ACTH preparations. Again the comparison of crude ACTH (Type IV) in gelatin with the purified ACTH (Type I) in gelatin shows good correlation with clinical findings and with other subcutaneous assay methods.

In order to study further the application of several assay methods to the problem of ACTH standardization, additional assays were conducted on several samples of purified ACTH (Type I) in gelatin and crude ACTH (Type IV) in gelatin. All samples were first assayed by the provisional U.S.P. adrenal ascorbic acid depletion method on hypophysectomized rats to a standard error of ±10% (150 to 200 rats per sample). For subcutaneous assays, doses were always given in terms of I.U. or U.S.P. Units, so that all preparations can be considered to be 100% of each other, by the intravenous assay technique, within the limits of error indicated above.

Table 1 gives the results of the assay of several lots of purified ACTH (Type I) in gelatin and several lots of crude ACTH (Type IV) in gelatin when utilizing adrenal ascorbic acid depletion following subcutaneous administration in the hypophysectomized rat. It may be noted that each female rats. The average lambda value in these experiments was 0.23 (0.20 to 0.31) so the individual assays are subject to a standard error of about ±16% and the means to a standard error of about ±8%. The purified ACTH (Type I) versus crude ACTH (Type IV) ratio of 3.2, therefore, exhibits close correlation with clinical results and with the other subcutaneous assay methods.

Table 3 gives the results of several assays on crude ACTH (Type IV) in gelatin assayed against the purified ACTH experimental standard by utilizing adrenal weight increase obtained in normal rats. Lambda values for these assays were 0.4 to 0.5 so the individual assays are subject to standard errors of about ±32% but the means are accurate to a standard error of about ±16%. By this method the relative subcutaneous versus intravenous potency of purified ACTH (Type I) in gelatin is about three times that of crude ACTH (Type IV) in gelatin.

TABLE 3. ASSAY OF SEVERAL ACTH PREPARATIONS, SUBCUTANEOUSLY ADMINISTERED, AND MEASURING ADRENAL WEIGHT INCREASE IN NORMAL RATS.

Type IV crude ACTH in gelatin lot	Number or rats		Potency ratio versus type I purified ACTH in gelatin experimental standard.
1	42		.28
1	48		.25
2	42		.37
U.S.P. standard in gelatin			
	42		.35
		Mean	.31

1.0 I.U. purified Type I ACTH in gelatin = 1.0/.31 = 3.2 I.U. crude Type IV ACTH in gelatin.

DISCUSSION

The results indicate that if crude ACTH (Type IV) in gelatin were assayed by a subcutaneous method against the present U.S.P. standard, about the same value would be obtained as at present with the intravenous assay procedure. If the purified ACTH (Type I) in gelatin were assayed against the U.S.P. standard by the same method, a relative potency of about three times that of the intravenous assay technique would be obtained. This corresponds to the clinical assay of these two types of ACTH preparations. The inference is that, if preparations were assayed against the present U.S.P. standard by a subcutaneous method, the resulting products would be uniform in clinical activity when labeled in terms of U.S.P. units and used subcutaneously or intramuscularly. The clinical doses in terms of U.S.P. units would then be the same for either preparation. If such preparations were now labeled in terms of U.S.P. units as determined by the provisional U.S.P. intravenous assay procedure, one U.S.P. unit of one preparation would be about three times as potent clinically as one U.S.P. unit of another preparation, even though each preparation utilizes a gelatin vehicle. ACTH preparations in gelatin equivalent by the provisional U.S.P. assay are, therefore, not necessarily equivalent clinically following subcutaneous administration.

Some evidence exists (Fisher, 1953) that effective ranges of 0.5 to 3 times the U.S.P. unit may be encountered when comparing different type preparations. An experiment was designed to determine whether differences in dose response curve slopes or differences in time response pattern would be detectable after intravenous administration of Type I and Type IV ACTH preparations. The method used was the same as the Munson *et al.* (1948) assay procedure with the exception that rats were sacrifieed at several time intervals. This experiment failed to show a difference between the two preparations comparable to that in the subcutaneous experiments. The time-response patterns appeared identical within limits of error. If a time difference in dose response curve slope exists, the purified Type I preparation shows the flatter slope. This latter feature is the opposite of findings after subcutaneous administration of the preparations. Comparison of the results of this experiment with the result of subcutaneous assays indicated that the major difference in relative potency of the two ACTH preparations appeared only after subcutaneous or intramuscular administration. The difference, therefore, must be due to a difference in rate of absorption or a difference in the rate of extravascular destruction.

The adrenal ascorbic acid depletion method, when ACTH is subcutaneously administered, discriminates quite sharply between preparations. The discrimination appears chiefly in the form of both deviation from parallelism of the log-dose response curve (Fig..2), and differences in time-response pattern (Fig. 1).

All characteristics of the thymus involution experiment in Figure 5 indicate that this could be the basis for a simplified and more economical assay for ACTH than any previously available. Precision appears excellent in spite of the absence of litter-mate distribution. The method also retains the feature of discrimination between preparations in respect to their relative subcutaneous potencies. While only results obtained from the Sprague-Dawley strain of rats have been reported, other strains, Wistar for example, have proven satisfactory.

Response range is adequate. Even for randomly selected rats, precision (lambda = 0.23) appears as good as, or better than, other methods. The use of litter-mates may increase precision.

Since there appears to be a difference in the relative potency of different

TABLE 1. AN ASSESSMENT BY 3 OBSERVERS OF THE DEGREE OF PROPTOSIS OF THE EYEBALLS IN A GROUP OF CORTISONE TREATED RATS AND THEIR CONTROLS

0 = No proptosis. x = Mild. xx = Moderate. xxx = Marked. xxxx = Gross.

Animal No.	Degree of proptosis present			Cortisone
	Observer			
	K.A.	S.M.G.	R.L.	
6	0	0	0	No
38	0	0	0	No
14	xx	xx	xx	Yes
7	0	x	0	No
13	xx	x	x	Yes
85	0	x	x	No
46	0	0	0	Yes
30	xxxx	xxxx	xxxx	Yes
47	x	x	0	Yes
55	0	0	0	No
23	0	0	0	No
29	xx	xxx	xxx	Yes
111	xxx	xxx	xx	Yes
112	x	0	0	Yes
77	0	0	0	No
51	0	0	0	No
104	xx	xx	xx	Yes
103	0	0	0	Yes
63	x	xx	x	Yes
22	0	0	0	No
39	xx	x	x	Yes
62	xx	xxx	xx	Yes
69	0	0	0	No

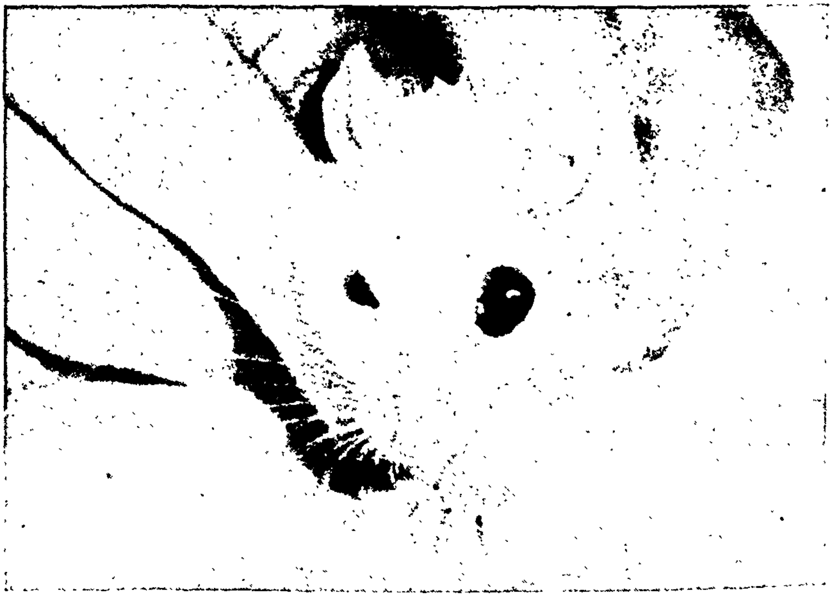

FIG. 2. Gross unilateral ocular protrusion of left eye with only mild proptosis of right eye in a male rat treated with cortisone.

metrical, and no other eye signs, such as the "bloody ring" described by Yudkin, Farquhar and Wakeman (1934) were observed. Proptosis was still present in many animals at the time of autopsy; in at least 4 animals, however, a previously established protrusion had disappeared.

Of particular interest was the course of exophthalmos in one animal, No. 30, to which reference has already been made. Proptosis was diagnosed in this animal by the 3rd day after the start of the cortisone injections. On the 11th day, nothing unusual was noticed, but on the 13th day, the left eye had literally left its socket (Fig. 2). Only moderate proptosis was present on the right side. The left eye as a whole appeared enlarged and close inspection revealed a certain degree of keratoconus. Of great interest was the occurrence here of the unilateral "bloody ring" noted by Yudkin *et al.* (1934) in some of their proptotic animals, a change which is presumably identical with the sanguineous discharge described by Thompson (1933). Pressure on the eyeball did not seem to cause any discomfort. The following day keratoconus was definite; the cornea had become completely dry and opaque; and the proptosis was unchanged. The picture was suggestive of a retroorbital tumour, and it was decided to kill the animal to avoid the possibility of a secondary infection preventing a correct diagnosis. The eye was punctured under light ether anaesthesia and clear fluid escaped under pressure, presumably from the anterior chamber. This undoubtedly was the cause of the keratoconus and the apparent enlargement of the eye, but it did not account for the proptosis, which still persisted. Enucleation showed that the eyeball was not appreciably enlarged. The retroorbital contents appeared hyperaemic, but no tumour or pus could be found. This was confirmed by histological examination (Fig. 3).

This peculiar picture was not observed in any other rat in this or any other series of similar experiments. It is possible that it is similar to the rare unilateral exophthalmos of endocrine origin in man.

DISCUSSION

Yudkin *et al.* (1934) observed proptosis in young male albino rats that were being given diets low in salts. Of 34 rats 11 developed marked and 11 slight and occasionally transient ocular protrusion. A "bloody ring around one eye" was noted in 4 animals. No explanation for this unilateral occurrence is given. On histological examination no evidence of increased ocular tension could be found, nor were there any changes present in either the eye muscles or the retrobulbar tissues. The thyroid is said to have shown "striking changes." Loss of hair was recorded in some animals, but no abnormality was reported in the pituitary or the adrenals, the weights of which, however, are not given.

Somewhat similar findings have been reported by Thompson (1933), who kept rats on diets with varying calcium and iodine contents. Ocular disturbances occurred in the form of proptosis and decreased frequency of blinking. Occasionally a sanguineous exudate developed around the lids of one or both eyes. Proptosis became noticeable about the sixth week; reached its maximum during the eighth to tenth week; and remained unaltered from then on. These eye changes were limited to animals on diets containing little iodine, in whom hyperplasia of the thyroid glands, followed later on in some cases by atrophy, was found. Apart from changes in the parathyroid glands the state of other endocrine organs is not mentioned.

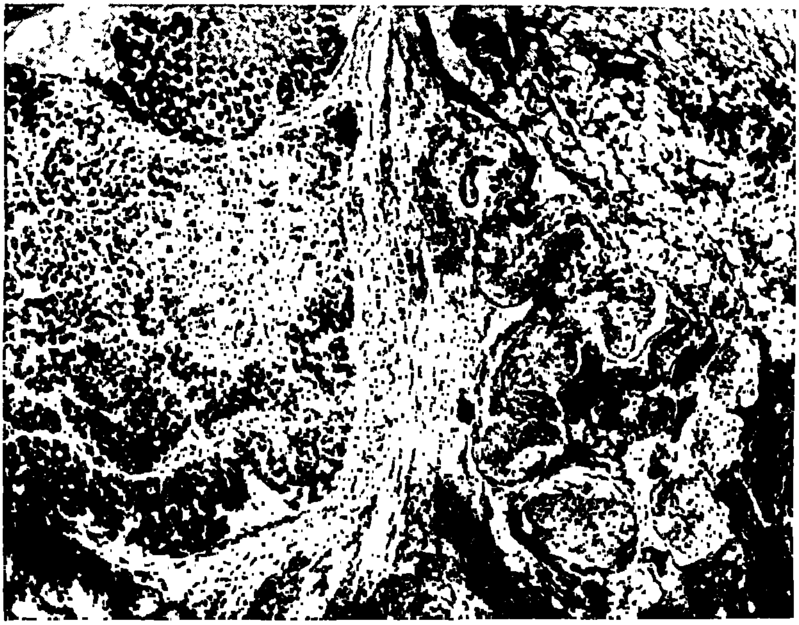

FIG. 3. Section of the left orbital tissues of the rat with asymmetrical proptosis. There is no evidence of inflammation or tumour present. (Formalin-Zenker, Hematoxylin-Eosin, $7\mu \times 75$).

Heinemann (1937) produced exophthalmos in guinea pigs and rats by injecting thyrotropic hormone (Schering-Kahlbaum). At autopsy the thyroids were shown to be enlarged. Exophthalmos occurred in animals treated for more than 2 weeks, and was most marked in those animals which had been treated longest. There was no loss of weight, but loss of fur was noted. The adrenal glands in the exophthalmic guinea pigs were definitely enlarged.

Definite exophthalmos persisting after death has been reported by Rawson, quoted by Means (1944), in rats in which a hyperplastic goitre was produced by treatment with sulfathiazole. Sellers and Ferguson (1949) have also observed proptosis in rats fed with propylthiouracil for prolonged periods. It could be seen in some animals after only 3 months, but it took 7 months before it was present in almost every rat. It was best observed if the animal was active and alert. All rats were under weight and had scanty, coarse hair. The weight of the eyeballs was not larger than in the control group, and no lymphocytic infiltration was present in the retrobulbar tissues.

It is generally stated that exophthalmos is due to excessive production of the thyrotropic hormone. The occurrence of thyroid changes in the experiments just quoted could be interpreted as supporting this thesis. Since the prolonged administration of cortisone or ACTH is said to induce thyroid depression—the "corticogenic hypothyroidism" of Wolfson *et al.* (1950, 1951)—the occurrence of proptosis in cortisone-treated animals could be explained on the basis of a subsequent increase in the secretion of the thyrotropic hormone. Some clinical (Richardson *et al.* 1950; Reiss *et al.* 1950; Werner, 1950); and experimental (Winter *et al.*, 1950) findings do not, however, support this view. Since it has furthermore been shown that cortisone significantly increases the thyrotropin-induced exophthalmos in normal as well as in thyroidectomised guinea pigs (Aterman, 1952b, c), it is difficult to maintain the thesis that this cortisone effect is mediated through the thyroid. Even if cortisone can affect the thyroid as an indirect consequence of some change in the pituitary, it is also probable that the hormone acts directly on the connective tissues of the body. The explanation of the ocular protrusion observed in the present experiments would then be the changed physical state of these tissues within the orbit (Aterman 1952b, c).

Reference has already been made to the unusual course of exophthalmos in rat No. 30 (Fig. 2). No explanation can be advanced for the exceptional features present in this animal. Since an increase, no change, or a decrease in the intra-ocular tension under the influence of ACTH or cortisone have been reported under various clinical and experimental conditions (Bardram, 1950; Fitzgerald *et al.* 1951; Olson *et al.* 1951; Sallman *et al.* 1952; Stewart Duke-Elder, 1951), it is questionable whether the increased tension present in the proptosed left eye of this rat can be attributed to a direct effect of cortisone or whether it was the result of the pronounced exophthalmos. The marked asymmetry of the latter also remains unaccounted for. Although some of the rats in the present series developed quite a marked degree of exophthalmos, we have not been able to reproduce a similar picture. Unilateral exophthalmos in experimental animals has, however, been reported by other workers (Friedgood, 1934; Smelser, 1937; Thompson, 1933). The absence of any macroscopic or microscopic evidence of a retroorbital tumor or inflammation (Fig. 3) in our experimental animal suggests a similarity with the rare but well known cases of "unilateral" endocrine exophthalmos in man occasionally mistakenly diagnosed as retroorbital tumors and even operated on (Anderson, 1947; Brain, 1936, Bedrossian, 1951; Burch, 1929, 1942; Denis and Mahaux, 1951; Drescher and Benedict, 1950; Jensen, 1940; Kisner and Mahorner, 1947; Klotz, 1948; Regnier, 1942; Rundle and Wilson, 1944, 1945; Saltzmann and Mellicker, 1951). That endocrine factors should lead to asymmetrical changes in symmetrical organs is a puzzling, but not unknown phenomenon of which several instances can be found in the literature (Bond, 1914; Greene, 1948; Peyton, 1942; Wilkins, 1948).

SUMMARY

The occurrence of varying degrees of bilateral proptosis has been observed in young male Wistar rats treated for 4 weeks with cortisone acetate. In one animal marked asymmetry of the proptosis occurred. These findings are discussed in the light of our present knowledge of the etiology of exophthalmos.

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NITROGEN BALANCE, LIVER PROTEIN REPLETION AND BODY COMPOSITION OF CORTISONE TREATED RATS

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IN 1940, Long, Katzin and Fry demonstrated that the administration of adrenal cortical extract to fasting rats caused a loss of nitrogen in the urine and an increase in liver glycogen. They suggested a relationship between protein breakdown and glycogen synthesis in such animals. For reviews of subsequent investigations, the reader is referred to those of Ingle (1950), Sayers (1950) and Engel (1951).

When cortisone acetate[1] became available in 1949, it seemed desirable to investigate further the effects of cortisone on nitrogen balance, on liver protein regeneration, and on the distribution of protein and fat in the body.

EXPERIMENTS AND RESULTS

Male albino rats of the Carworth strain were selected from single shipments and grouped to obtain equivalent average body weights. Adrenalectomies were performed by the lumbar approach under nembutal anesthesia and operated rats were given 1% saline to drink. All animals were housed in individual mesh-bottom cages in air conditioned quarters during the experiments. Nitrogen was determined by the micro-Kjeldahl method. References to other analytical techniques accompany the data.

The probability (p) of erroneously concluding that there are significant differences between means was calculated by Student's t test or from the variance ratio (F). See Snedecor (1946).

a) *Nitrogen balance.* Rats weighing 250 gm., on the average, were fed protein-free diet (Silber and Porter, 1950), *ad libitum* for 7 days. On the 8th, 9th, and 10th days, consumption of the diet was limited to 8 gm./rat /day and each of thirty-two rats was given daily subcutaneous injections of 3 mg. cortisone acetate in aqueous suspension. A second group of thirty-two rats did not receive cortisone. On the 9th day, all rats were placed in metabolism cages in groups of two. On this and the subsequent day, half of the cortisone-treated rats and half of the untreated rats each received, in addition to the daily allotment of protein-free diet, an oral dose of a solu-

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[1] Cortisone Acetate (Merck).

tion of amino acids or egg albumin (300 mg. N/kg. rat). Twenty-four hours after the second dose, the rats were given 10 ml. of water orally, and urines were collected with cage washings two hours later. The urinary nitrogen data are summarized in Table 1.

TABLE 1. THE EFFECT OF CORTISONE ACETATE UPON NITROGEN BALANCE. NITROGEN GIVEN, 300 MG./KG./DAY

Group	Treatment		Av. rat wts. before test, gm.	3-day wt. loss, gm.	Liver wt., % body wt.	Av. urinary N, mg./kg./day ± s.e.
	Corti-sone	Amino acids				
			Amino acid experiment			
1–4	+	+	226	11.5	4.1	474 ± 7.2
5–8	+	0	225.5	15.5	3.7	365 ± 24.1
9–12	0	+	227	2.0	3.2	372 ± 5.4
13–16	0	0	226	8.0	3.0	267 ± 9.7
			Egg albumin experiment			
	Corti-sone	Egg albumin				
1–4	+	+	243	—	—	367 ± 7.2
5–8	+	0	239	—	—	338 ± 25.0
9–12	0	+	239	—	—	272 ± 17.3
13–16	0	0	238	—	—	231 ± 6.4

Sixty-four per cent of the orally administered amino acid nitrogen was retained by the cortisone-treated rats and 65% by the controls. The retention of egg albumin nitrogen also was comparable in the two groups, 90% and 86%, respectively. Expressed as nitrogen balance indices (Allison, Seeley, and Ferguson, 1947) these values would be 0.64, 0.65, 0.90, and 0.86. Thus, cortisone had no effect on the retention of ingested nitrogen. The rats lost 13 gm. body weight/kg. and 100 mg. urinary nitrogen/kg./day more than their controls. Nevertheless, the livers of the rats given cortisone contained 200 mg. more nitrogen/kg. body weight than those of the controls.

b) *Liver protein repletion.* Rats which were adrenalectomized on the fourth day of protein depletion were supplied protein-free diet for six additional days. On the 11th, 12th, and 13th days some were allowed to consume 4.5 gm. protein-free diet, others 4.5 gm. 20% casein diet per 100 gm. body weight each day. Cortisone injections (1.5 mg./100 gm. body wt.) were made daily from the fourth to the thirteenth day, inclusive. Intact rats were treated similarly.

No significant difference was observed between the intact and the operated rats so the data were pooled, as shown in Table 2. The livers of the repleted, cortisone-treated rats were larger and contained more protein nitrogen, more NPN and more glycogen. Serum protein concentrations

TABLE 2. THE EFFECTS OF CORTISONE ON LIVERS OF RATS DURING PROTEIN-REPLETION

	Cortisone-treated (13 rats)	Controls (9 rats)
Liver wt., gm./100 gm. rat	5.28 ± 0.16	3.18 ± 0.061
Protein N. mg./100 gm. rat	127.8 ± 1.7	94.6 ± 2.0
N. P. N., mg./100 gm. rat	17.9 ± 0.35	13.0 ± 0.27
Glycogen, mg./100 gm. rat	429.8 ± 36	48.8 ± 5.9

were also increased (6.6 versus 5.7 gm.%). Similar but less striking differences were seen in the rats which remained on protein-free diet throughout the experiment.

The subsequent experiment was designed to study the quantitative relationship between the effects of cortisone and protein feeding upon liver protein regeneration. Adrenalectomized rats were fed protein-free diet *ad libitum* from the fourth through the seventh day after operation. They were then allowed to consume 5 gm. per 100 gm. body weight per day of protein-free or 15% casein diet, and some were given daily subcutaneous injections of cortisone acetate (2 mg./100 gm. rat). Rats were sacrificed two or four days after the first injection. Analysis of variance of the data (Table 3) shows that the very significant effects of cortisone and of dietary protein upon liver nitrogen had no influence upon one another.

c) *Effects of cortisone on carcass and viscera of rats.* From the above results, it was clear that cortisone could cause an increase in liver and serum protein while nitrogen was being lost in the urine, whether or not the animals were supplied dietary protein. The following experiment was designed to locate the source of the endogenous nitrogen available for liver regeneration and for excretion.

Five days after adrenalectomy, groups of five rats were injected daily with 0.1 or 1.0 mg. cortisone acetate per 100 gm. rat, and allowed to consume 3.5 gm. of protein-free diet per 100 gm. rat/day. Control animals were not included because adrenalectomized rats do not survive on the protein-free diet in spite of saline therapy.

The rats were sacrificed after 14 days. They were exsanguinated, and the carcasses were divided as follows: (1) liver, (2) spleen, (3) gastro-intestinal organs (washed stomach and intestines, and adhering structures), (4) urogenital organs (testes, prostate, kidneys, bladder), (5) pleural organs (heart and lungs), (6) carcass (muscle and bone, including the head and brain), and (7) skin and hair.

Livers and spleens were homogenized with water and aliquots taken for the various analyses. The other tissues were macerated in a Waring Blender with 70% alcohol, then extracted in Soxhlet extractors with 95% ethanol, then ether. The extracts were evaporated to dryness and the residues

TABLE 3. EFFECT OF PROTEIN AND CORTISONE UPON LIVER N REGENERATION IN ADRENALECTOMIZED RATS

5 rats/group; saline *ad libitum*

Protein	Cortisone	Days	Body wt. at start, gm.	Body wt. at sac., gm.	Liver N, mg./100 gm. rat ±s.e.
0	0	2*	190	178	85.9 ±2.76
0	0	4*	190	168	84.3 ±1.40
0	+	2	190	181	101.8 ±1.18
0	+	4	190	172	110.1 ±3.39
+	0	2	190	189	99.0 ±2.84
+	0	4	190	193	102.4 ±2.06
+	+	2	190	183	126.4 ±1.68
+	+	4	190	186	125.3 ±3.50
		0	190	190	92.2 ±2.40

* These groups consumed 3.5 and 2.5 gm. diet/100 gm. rat/day. The remaining groups consumed all 5 gm. of their diets/100 gm. rat/day.

ANALYSIS OF VARIANTS

Source of variation	Degrees of freedom	Mean squares	F*	p
Protein	1	3766.57	100.79	<0.005
Cortisone	1	6352.60	169.99	<0.005
Days	1	59.86	1.60	
Protein × Cortisone	1	56.77	1.52	
Protein × Days	1	14.95	0.40	
Cortisone × Days	1	21.60	0.58	
Protein × Cortisone × Days	1	156.24	4.18	0.05
Error	40	37.37		

* F for 1 and 40 d.f., ($p=0.005$) $=8.83$; ($p=0.05$) $=4.08$.

partitioned between water and petroleum ether. The defatted tissues were dried and pulverized, and aliquots were taken for analysis, except in the case of skin, where the whole sample was used. For protein analyses samples of dried defatted carcasses and the skins were extracted with N/10 NaOH. Three 24-hour extractions at room temperature were sufficient to remove essentially all of the soluble proteins from the carcass samples. The skins were extracted four times, with freezing and thawing, to remove the soluble proteins. Collagen in carcasses and skins was determined essentially according to Lowry, Gilligan and Katersky (1941). Total nitrogen on the residues from the collagen extractions of the carcasses is reported here as "elastin" N; the figure also includes bone nitrogen. Since the hair was not separated from the skins, total nitrogen on skin residues was not determined.

The rats which received the higher dose of cortisone lost significantly more weight than those receiving the lower dose (Table 4). The carcasses and skins lost weight roughly in proportion to body weight. The internal organs maintained or increased in weight, so the organ weight to body weight ratios showed significant increases. The analytical data on blood

TABLE 4. ORGAN WEIGHTS, SERUM PROTEIN AND LIVER ANALYSES. ADRENALECTOMIZED PROTEIN-DEPLETED RATS TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	p
Initial body wt., gm.	229	229	
Body wt. loss, %	27.3 ± 0.96	30.8 ± 0.33	<0.025
Carcass wet wt., gm./100 gm. rat	60.2 ± 0.83	58.0 ± 0.59	<0.1
Dry, defatted, carc. wt., gm./100 gm. rat	16.8 ± 0.20	16.4 ± 0.38	>0.2
Skin and hair wet wt., gm./100 gm. rat	18.1 ± 0.38	17.3 ± 0.21	<0.2
Dry, defatted skin wt., gm./100 gm. rat	6.08 ± 0.18	6.06 ± 0.08	>0.2
Liver wt., gm./100 gm. rat	2.93 ± 0.07	4.18 ± 0.12	<0.005
Liver dry wt., gm./100 gm. rat	0.75 ± 0.022	1.14 ± 0.042	<0.005
Viscera wt., gm./100 gm. rat	11.3 ± 0.29	12.7 ± 0.51	<0.05
Dry, defatted viscera wt., gm./100 gm. rat	1.31 ± 0.024	1.46 ± 0.055	<0.05
Total serum protein, gm. %*	5.27 ± 0.16	6.00 ± 0.071	<0.005
Albumin (pooled sample), gm. %	1.45	2.12	
Globulin (pooled sample), gm. %	3.72	3.93	
A/G ratio	0.39	0.54	
Liver protein N, mg./100 gm. rat	76.5 ± 1.31	105.5 ± 1.86	<0.005
Liver NPN, mg./100 gm. rat	9.47 ± 0.30	13.51 ± 0.34	<0.005
Liver carbohydrate, mg./100 gm. rat	137 ±23.4	320 ±20.5	<0.005
Liver lipid (alc.-ether sol.) mg./100 gm. rat	261 ±22.3	452 ±24.6	<0.005
Liver desoxyribonucleic acid, mg./100 gm. rat†	15.2 ± 0.39	17.1 ± 1.26	<0.2
Liver ribonucleic acid, mg./100 gm. rat‡	43.8 ± 3.56	68.4 ± 4.10	<0.005

* Total protein and protein fractions by the method of Kibrick and Blonstein (1948).
† Dische (1930).
‡ Davidson and Waymouth (1944).

serum showed that cortisone prevented a decrease or caused an increase in albumin, even though these animals were consuming protein-free diet, but there was little change in globulin.

As the livers increased in size under the influence of the higher dose of cortisone, protein and fat (alcohol-ether soluble) increased in about equal proportions while carbohydrate increased to a greater extent. Liver ribonucleic acid increased in proportion to liver weight, but desoxyribonucleic acid remained essentially constant in quantity, indicating an increase in cytoplasm without an increase in the number of cells. (The relative increase in desoxyribonucleic acid indicated in the table was of doubtful significance, $p < 0.2$, and was at least partially a result of the decrease in body weight.)

The distribution of nitrogen in the animals (Table 5) closely paralleled the organ and body weight distribution discussed above (Table 4). Total nitrogen of the rats decreased proportionately with body weight loss. However, there was an excessive loss of nitrogen from the carcass whereas the liver, gastro-intestinal organs and urogenital organs were spared nitrogen loss. The collagen and elastin nitrogen of the carcass showed a relative and absolute increase, while the more labile alkali-soluble nitrogen fraction decreased significantly. The fraction of nitrogen contributed by the skin remained the same in the two groups.

The ratio of total fat (Table 6) to body weight increased by 50%

TABLE 5. NITROGEN DISTRIBUTION IN ADRENALECTOMIZED, PROTEIN-DEPLETED RATS TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	p
Nitrogen, % of rat total N (skin elastin and hair omitted)			
Carcass	68.0 ±0.49	65.0 ±0.43	<0.01
Skin	22.1 ±0.45	22.6 ±0.32	>0.2
Liver	3.03 ±0.085	4.49 ±0.114	<0.005
Spleen	0.30 ±0.055	0.25 ±0.019	>0.2
Pleura	0.25 ±0.004	0.25 ±0.007	>0.2
Gastro-intestinal	3.66 ±0.070	4.27 ±0.130	<0.01
Urogenital	2.55 ±0.073	2.96 ±0.120	<0.05
Total N gm./100 gm. rat	2.79 ±0.061	2.67 ±0.037	>0.2
N of carcass fractions, % of carcass total N			
Water solubles from Soxhlet extract	5.84 ±0.28	6.77 ±0.27	<0.1
Alkali soluble	82.30 ±0.57	76.87 ±0.48	<0.01
Collagen	10.16 ±0.54	14.24 ±1.00	<0.025
Elastin & bone	1.67 ±0.088	2.11 ±0.12	<0.05
Carcass total N, gm./100 gm. rat	1.90 ±0.053	1.74 ±0.031	<0.1
N of skin fractions, % of skin total N (hair & elastin omitted)			
Water soluble from Soxhlet extract	6.84 ±0.34	5.84 ±0.33	<0.2
Alkali soluble	41.56 ±0.53	41.16 ±1.12	>0.2
Collagen	51.48 ±0.88	52.90 ±1.17	>0.2
Soluble N+Collagen N, gm./100 gm. rat	0.62 ±0.017	0.61 ±0.001	>0.2

($p < 0.1$) under the influence of cortisone. Particularly striking increases in fat were observed in the carcass and organs of the pleural cavity and less striking increases in the urogenital organs and livers. Fat in the skin and gastro-intestinal organs remained essentially constant.

DISCUSSION

Since the report of Long, Katzin and Fry (1940) cortisone has frequently been termed a "catabolic" hormone. From isotope experiments, Hoberman (1950) and Clark (1950) have considered cortisone to exert catabolic or

TABLE 6. FAT* IN ADRENALECTOMIZED PROTEIN-DEPLETED RATS. TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	*p*
Carcass, gm. fat/100 gm. rat	1.46 ±0.14	2.62 ±0.35	<0.025
Skin, gm. fat/100 gm. rat	0.91 ±0.22	1.03 ±0.09	>0.2
Liver, mg. fat/100 gm. rat	115 ±11.4	158 ±20.8	<0.2
Pleura, gm. fat/100 gm. rat	41.5 ±5.9	95.3 ±11.8	<0.005
Gastro-intestinal, mg. fat/100 gm. rat	317 ±159	588 ±137	>0.2
Urogenital, mg. fat/100 g. rat	451 ±101	731 ±81	<0.1
Spleen, mg. fat/100 gm. rat	14.2 ±2.5	16.3 ±1.3	>0.2
Total fat, gm./100 gm. rat	3.30 ±0.62	5.24 ±0.63	<0.1

* Benzin soluble.

anti-anabolic effects on protein metabolism. Ingle (1950) and Engel (1951) have suggested that the primary effect of cortisone may be a decrease in the utilization of carbohydrate. The fact that cortisone in milligram quantities can depress the body weight of rats without a proportionate decrease in food intake is well substantiated. From the observations of Kochakian and Robertson (1951) and from the present work, it is clear that the loss of body weight is due to loss of substance, including nitrogen, from the carcass rather than from the internal organs.

Loss of nitrogen from the carcass was not accompanied by deficient utilization of ingested protein as evidenced by nitrogen balance and liver protein repletion. This is in agreement with the report of Wells and Kendall (1940) that adrenalectomy did not influence the utilization of exogenous protein.

Engel, Schiller, and Pentz (1949) found that in nephrectomized rats, exogenous amino acids or glucose spared the breakdown of endogenous protein caused by injection of adrenal cortical extract. In the present experiments, however, the effect of cortisone upon the incorporation of nitrogen in the liver or its excretion in the urine was not influenced by the ingestion of minimal amounts of protein by intact rats. In rats fed normal amounts of dietary protein Clark (in press) found that cortisone treatment caused an increase in the excretion of isotopic nitrogen fed as glycine. The apparent contradiction between this and the present work may be related to the level of nitrogen intake.

In the nitrogen balance experiment it was noted that the body weights of the rats given cortisone decreased 13 gm./kg./day more than their controls in spite of equivalent caloric intakes. If the tissue protein is assumed to contain 16% nitrogen and the wet tissue to contain 20% protein, only one-fifth of the weight loss can be related to the loss of nitrogen in the urine. There was no evidence in these experiments that dehydration occurred in the carcasses of the cortisone-treated rats although they did excrete larger volumes of urine. They also consumed more water, and dry weight data showed that the water concentrations in the carcasses were comparable in the treated and untreated groups.

The liver protein of cortisone-treated rats decreased in concentration, but increased in total, This may be related to the deposition of glycogen and the increase in cell size. However, the elevated serum albumin concentrations found in the cortisone-treated rats also indicates an increase in protein synthesis by the liver. Mushett, Porter and Silber (unpublished) have found that cortisone-treated dogs on stock diet *ad libitum* showed changes similar to those observed in rats, i.e., in liver protein, fat, and nucleic acid. Friedgood, Vars and Zerbe (1950) have reported that cortisone facilitated liver regeneration after partial hepatectomy. Kochakian and

Robertson (1951) called attention to the fact that protein anabolism may occur in certain tissues simultaneously with catabolism in others. It seems reasonable to conclude that the protein catabolic (or anti-anabolic) effects of cortisone were, under the conditions of our experiments, essentially limited to the labile carcass proteins.

SUMMARY

In nitrogen balance and liver protein repletion experiments cortisone did not alter the biological value of an amino acid mixture, egg albumin or casein.

In protein-depleted rats, cortisone caused a wastage of nitrogen from carcass fractions other than structural protein (collagen and elastin). Concurrently, the total protein of the liver, gastro-intestinal tract and urogenital organs, as well as plasma albumin were increased. The increased size of livers caused by cortisone may be due primarily to a laying down of cytoplasm (ribonucleic acid increased) without the formation of new cells (desoxyribonucleic acid not increased). An increase in total fat of the animal was observed, with the most significant increases occurring in the carcass and organs of the pleural cavity.

Acknowledgment

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RADIOIODINE METABOLISM IN THE CHICK EMBRYO

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INTRODUCTION

MANY studies have been made of the histology of the thyroid gland at various stages in the development of the chick embryo. These studies, as well as several to determine the age of the chick embryo at which biochemical differentiation or thyroid function begins, have recently been summarized by Moore (1950). Methods used to determine thyroid function in the chick embryo include measurement of the total iodine in the thyroid glands (Sun, 1932), demonstration of the presence of the thyroxine in the chick thyroid by tadpole test (Hopkins, 1935), and demonstration of organic binding of radioiodine in the thyroid gland by autoradiography (Hansborough and Khan, 1951). Using these methods, it was not possible to detect any evidences of thyroid function or biochemical differention prior to the tenth day of incubation, which is the time colloid has been reported to appear in the thyroid gland (Bradway, 1929; Hopkins, 1935; Venzke, 1949).

It has been recognized that in addition to its ability to incorporate iodide into organic binding and to synthesize thyroxine, the thyroid gland differs from non-thyroid tissue in that it has the property of maintaining a concentration of iodide greatly elevated above that of the blood (Albert, 1952).

The investigation reported in this paper was undertaken to determine the temporal relationships involved in the development of the ability of the embryonic thyroid gland to maintain a concentration of iodide above that of blood and the ability to incorporate iodide into organic binding. Radioiodide and quantitative counting procedures were used.

It was found that the ability of the thyroid gland to concentrate iodide from the blood develops before its ability to incorporate iodide into organic binding. Iodide concentrating occurred before follicle formation at a stage when there was not more than a trace of colloid in the gland. A few data have also been obtained on the distribution of I^{131} in some of the major components of the egg within 2 hours after injection of radioiodide.

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MATERIAL AND METHODS

These studies were made on eggs from White Leghorn chickens. Incubation for the required interval was at 37.5° C. in a forced draft incubator. The eggs had been brought to room temperature before they were placed in the incubator, and no allowance was made for a "warm up" period; age was computed from the time that the eggs were placed in the incubator. The embryos corresponded to the stages described by Hamburger and Hamilton (1951). The thyroid gland morphology corresponded to the appropriate stages described by Venzke (1949).

Forty microcuries of I^{131} in the form of 0.05 ml. of carrier-free NaI was injected into one of the allantoic veins by means of a fine cannula sealed into a 0.1 ml. serological pipette. The window in the shell was sealed with "scotch" tape and the eggs were placed in a still air incubator until needed. At appropriate intervals they were reopened and a venous sample (0.05 ml.) was obtained and delivered into 0.95 ml. of H_2O containing 1.0 mg. of NaI. Immediately after the blood was taken the embryo was removed from the egg. Every effort was made to drain any extraneous fluids from the carcass. It is clear, from the values obtained for the I^{131} concentration in possible contaminating fluids (Table 2), that such contamination could not make an appreciable contribution to the concentration of I^{131} in the carcass. In no case was a carcass contaminated with yolk. Thyroid glands were dissected out, measured with an ocular micrometer and then placed into either Bouin's solution or an homogenizer containing 4 ml. of ice-cold H_2O to which 1.0 mg. of NaI carrier had been added. The weights of the thyroid glands were calculated by computing their volume from the lengths of their major axes and assuming a density of one. The carcass below the neck was weighed and put into a sample bottle or an homogenizer which contained 5 ml. ice-cold 8% TCA.

Complete serial sections were made of thyroid lobes from several 7-, 9- and 10-day embryos. Sections were stained with hematoxylin and eosin. Later the sections from 3 of the 7-day embryos and 4 of the 9-day embryos were destained and then treated with periodic acid-Schiff reagent to test for colloid.

I^{131} in the diluted blood was determined by beta counting of a dried aliquot. Self absorption corrections were found to be negligible. Protein binding of I^{131} in the carcass was tested by homogenization in 8% TCA, centrifugation and determination of I^{131} in both the supernatant and the sediment by gamma counting. Protein binding of I^{131} in the thyroid glands was assessed after homogenization, deproteinization with the $Ba(OH)_2$-$ZnSO_4$ reagent of Somogyi (1945) and centrifugation.[2] I^{131} in the sediment was measured by gamma counting, that in the supernatant by beta or gamma counting. Some of the glands were dried and I^{131} was determined in them by beta counting. Although results using this technique agreed reasonably well with those using homogenized material only data from the latter are presented in the tables because of uncertainty of self-absorption corrections.

In a few 7-day eggs I^{131} determinations were made on allantoic fluid, amniotic fluid, blood, carcass below the head, fluid yolk and solid yolk. Samples for this survey were taken at approximately 75 minutes after injection.

RESULTS

The histology of the hematoxylin-eosin stained sections of the thyroid glands of the embryos studied (Figs. 1–3) appeared to duplicate observa-

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[2] Under the conditions used Somogyi's reagent has been found to precipitate organic bound I^{131} more completely than does TCA, although the reagent does not precipitate an appreciable amount of radioiodide (Wollman and Scow, unpublished results).

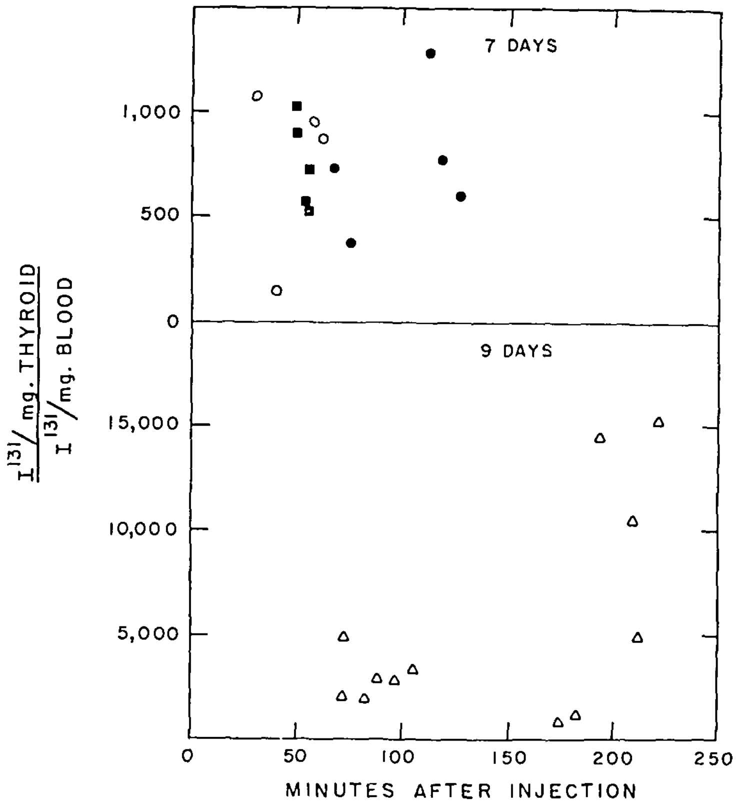

FIG. 6. The ratio of I^{131} content per mg. of thyroid gland to the I^{131} content per mg. of blood at various time intervals after injection of radioiodide. Data are shown for seven- and nine-day embryos. The three different symbols used in the seven-day data represent different experiments.

TABLE 1. THE RATIO OF I^{131} CONCENTRATION IN CARCASS AND THYROID GLAND TO I^{131} CONCENTRATION IN BLOOD OF CHICK EMBRYOS

Days incubated	Number of eggs	Time interval injection to sampling, minutes	*Estimated* thyroid weight, mg.	Average carcass weight,[1] gm.	Per cent dose in thyroid gland	$\frac{I^{131}/\text{mg. carcass}}{I^{131}/\text{mg. blood}}$	$\frac{I^{131}/\text{mg. thyroid}}{I^{131}/\text{mg. blood}}$ Total	Free
9	12	71 to 223	0.16	1.0	0.26 (12)	0.75 ± .04[2] (10)[3]	5400 ± 1200 (12)	400 ± 200 (3)
7	6	30 to 65	0.021	0.23	0.024 to 0.11 (3)	0.95 ± 0.07 (5)	700 (4)	700 (4)
	6	50		0.24	0.022 (5)	0.82 ± 0.05 (6)	700 (5)	700 (5)
	9	68 to 136		0.26	0.0037 to 0.068 (5)	0.80 ± 0.06 (9)	400 ± 106 (5)	400 ± 106 (5)

[1] Body below neck.
[2] Mean ± standard error.
[3] Number of eggs on which datum is based.

concentration of the carcass I^{131} averaged only 0.75 times that of blood and all of it was non-precipitable (Fig. 7).

In the 7-day embryos only .004 to .11% of the injected I^{131} was found in the thyroid gland. The radioiodine content was higher when the sampling was done relatively soon after the injection and lower when the sampling was delayed. No precipitable I^{131} was detected in the 7-day embryo thyroids. The ratio of the I^{131} content of the thyroid gland to the I^{131} content per mg. of blood was 15 ± 4. The concentration of I^{131} in these glands averaged 400 to 700 times that of blood (Fig. 6). The average value of the ratio was relatively constant during the time interval between 30 to 130 minutes after injection although measurements indicated that the I^{131} concentration of the blood at the end of this time interval could be only a few per cent of that at 30 minutes. The average concentration of I^{131} in the carcass varied from 0.80 to 0.95 times that of blood (Fig. 7) and was also independent of the time interval after injection. All the carcass I^{131} was non-precipitable.

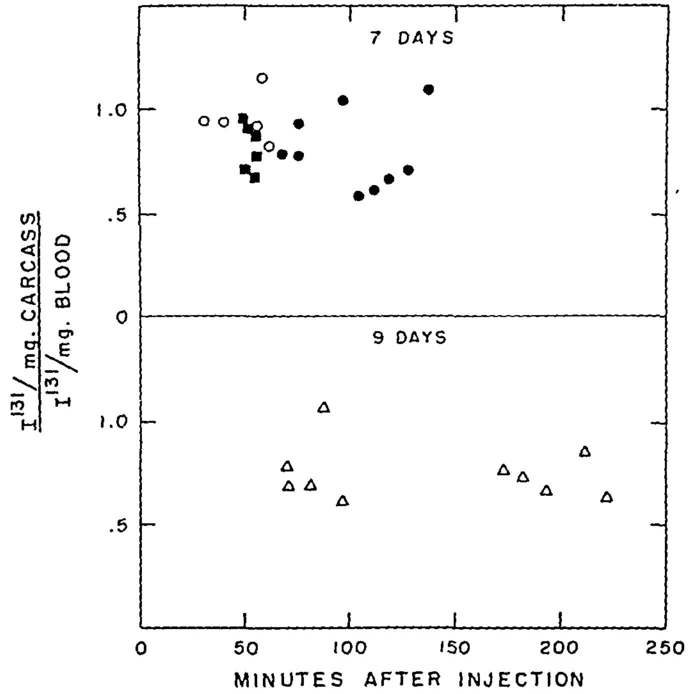

FIG. 7. The ratio of I^{131} content per mg. of carcass to the I^{131} content per mg. of blood at various time intervals after injection of radioiodide. Only the body below the neck was used in carcass determinations of these 7- and 9-day embryos. The three different symbols used in the seven-day data represent different experiments.

TABLE 2. DISTRIBUTION OF I^{131} AMONG SOME COMPONENTS OF THE CHICKEN EGG INCUBATED FOR SEVEN DAYS[1]

Component	Sample volume, ml.	Activity per ml., counts/sec/ml.	Total component volume,[2] ml.	Total activity in component, counts/sec.	Per cent dose in component	Activity per ml. / Activity per ml. if uniform distribution of I^{131}
Allantoic fluid	1.0	5,070 3,750	0.9	3,900	1.4	0.80
Amniotic fluid	0.05	1,670 1,870	1.3	2,300	0.80	0.33
Fluid yolk	1.0	14,900 13,500	13.0	185,000	65.0	2.6
Solid yolk	2.0	41 1,030	18.0	9,000	3.0	0.09
Head	0.406 0.473	1,930 1,520	0.41 0.47	785 716	0.26	0.32
Body	0.230 0.236	1,540 1,090	0.23 0.23	354 246	0.11	0.24
Total Egg		5,400	53.0	286,000	100.00	
Remainder of Egg[3]		4,500	19.0	85,000	29.7	0.83

[1] Approximately 75 minutes after injection of radioiodide.

[2] Volume of allantoic fluid and amniotic fluid from Romanoff and Hayward (1943). Volume of fluid yolk and solid yolk from Romanoff (1943).

[3] Data calculated by difference.

One of the striking findings in the initial experiments was that the concentration of I^{131} in the blood, carcass or allantoic fluid was less than would be expected if the injected I^{131} were uniformly distributed in the egg. In an exploration of a few 7-day eggs it was found (Table 2) that a major concentration of the I^{131}, both in regard to per cent of total dose and to relative activity occurred in the fluid yolk, which had ten times the concentration found in the carcass. The amniotic fluid had approximately the the same I^{131} concentration as the carcass while that of the allantoic fluid was somewhat higher. A number of components (e.g., yolk-sac membrane, albumen) were not tested and it is possible that a considerable concentration of I^{131} may have been present in one of these sites. For example, if it can be assumed that no I^{131} was present in the shell, the average I^{131} concentration in albumen and membranes would be half that in fluid yolk.

DISCUSSION

The difference in the results using 7-day and 9-day eggs cannot be attributed to either differences in the batches of fertile eggs used for the 7-

day and for the 9-day eggs, nor to differences in the composition of the stock radioiodide solution. The data listed in the last line of Table 1 and in solid circles in Figures 6 and 7 were obtained on eggs from the same batch as the 9-day eggs. Aliquots of the same stock radioiodide solution were used.

The data obtained in the present investigation show that the thyroid gland of the 7-day chick embryo has undergone biochemical differentiation to the extent that it already possesses the ability to maintain a concentration of non-precipitable I^{131} grossly elevated above that of the blood. The fact that the ratio of the concentrations of I^{131} in thyroid gland and blood was relatively constant while the I^{131} concentration of the blood was dropping suggests relatively free exchange and equilibration of the I^{131} in thyroid gland and blood. The capacity to incorporate iodide into organic molecules precipitable with Somogyi's reagent could not be demonstrated in these thyroid glands. If organic binding occurs it must be at a much slower rate than in the thyroid glands of the 9-day embryos which were found to have the capacity to incorporate radoiiodide into organic binding, in addition to the capacity to concentrate radiodide.

The above indications of biochemical differentiation both appear prior to the development of follicles in the chick embryo. It has been possible to demonstrate colloid-like material with the periodic acid-Schiff method in the stage in which organic binding of radioiodide was detected (9-day thyroids) and to demonstrate an almost complete absence of this material when radioiodide binding was not detected. In contrast, organic-bound iodine has been reported to occur prior to the appearance of both follicles and colloid in the thyroid gland of the fetal pig (Rankin, 1941) and the fetal calf (Koneff, Nichols, Wolff and Chaikoff, 1949).

There is an apparent contradiction between the present data demonstrating that the thyroid glands of the 9-day embryo have the capacity to bind iodide, and the work of Sun (1932), Hopkins (1935), and Hansborough and Khan (1951), who were not able to demonstrate organic bound iodine before the tenth or eleventh day of incubation. This contradiction may not be a real one. The present data demonstrate a qualitative capacity to incorporate available radioiodide into organic binding. There is no reason to believe that the radioiodide which was bound in the thyroid gland had equilibrated with endogenous iodide in the egg, or that endogenous iodide was present in the blood of the embryo and available for the thyroid gland to concentrate and bind. Sun's (1932) measurements on the total iodine concentration of the thyroid gland of the 9- and 10-day embryos when compared with the present data suggest that either endogenous egg iodine is not available to the embryonic thyroid gland or that the actual rate of binding of available iodide is quantitatively slow on the ninth day.

Hansborough and Khan's (1951) radioautograph studies (in which the radioiodide was injected into fertile eggs prior to incubation, and in which case it might be safe to assume that the radioiodide had equilibrated with endogenous iodide) suggest that the per cent of the endogenous iodide that is bound in the thyroid gland is too small to detect by their method prior to 11 days of incubation.[3]

No attempt has been made to determine the stage at which protein binding of radioiodide begins. This may be a difficult thing to do for, as shown by Wolff, Chaikoff and Nichols (1949), in the calf the concentration of bound thyroid iodine increases gradually but continuously throughout the period of fetal life which they studied. If this is so in general, the fixed sensitivity of radioautograph procedures as conventionally done may not be entirely satisfactory for determining onset of function, since if the sensitivity is fixed, i.e., if the dose of I^{131}, exposure time, and the sensitivity of the film are fixed, the measurements will probably give too late a time interval for onset of binding. For, if thyroid activity increases gradually during fetal life, increases in the sensitivity of the procedure might be expected to allow detection of a positive radioautograph at progressively earlier stages in development.

Removal of injected radioiodide from the blood and equilibration between the blood and the carcass were rapid. However, the concentration of I^{131} in the various parts of the egg was not uniform. In the 7-day embryos only the fluid yolk had a concentration greater than that expected from a uniform distribution of I^{131} throughout the egg contents. The concentration of I^{131} in the fluid yolk was ten times that of the blood, suggesting that the yolk-sac membrane has an active concentrating mechanism for iodide.

SUMMARY

Radioiodide was injected into an allantoic vein of 9-day and 7-day chick embryos. Quantitative determinations of I^{131} in thyroid gland, carcass, and blood were made using Geiger tubes. In a few embryos fluid and solid yolk, allantoic fluid and amniotic fluid were also assayed.

The thyroid glands of both the 9- and 7-day embryos concentrated

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[3] After this manuscript was submitted for publication two papers became available to us which support and supplement these remarks. Stoll and Blanquet (Annales d'Endocrinologie, 13: 569. 1952) found that six hours after radioiodide was administered to a 7-day egg the I^{131} content per unit weight of the thyroid was higher than that of the heart. In contrast, Blanquet, Stoll and Capot (C. R. Soc. de Biol. 146: 1103. 1952) found that when radioiodide was administered to laying hens 100 per cent of the I^{131} in the 7-day egg was found in the yolk plus albumen. No I^{131} was found in the embryo until the eighth day, and none in the thyroid until the tenth day.

I^{131}. In 9-day thyroids, the I^{131} concentration averaged 5000 times that of blood, and most of this was organic bound. The I^{131} concentration in 7-day glands was 400 to 700 times that of the blood, and almost all appeared to be non-precipitable and freely exchangeable with blood I^{131}. I^{131} concentration in the carcass was about 0.7 to 0.9 times that in blood.

The fluid yolk accumulated the greater proportion (65%) of the total dose within a relatively short time (1.5 hours).

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EFFECTS OF ANDROGENS AND ESTROGENS ON β-GLUCURONIDASE IN INBRED MICE[1,2]

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IN RECENT years, Morrow *et al.* (1949, 1950) reported that certain pure inbred strains of mice exhibited strikingly low concentrations of β-glucuronidase in the liver, spleen and kidney. Cohen and Bittner (1951) found that mammary tissue β-glucuronidase activity bore a relationship to the strain of mice employed, extremely low concentrations being found in the C3H strain .

Among the interesting questions which these findings provoked is the one of whether or not tissue β-glucuronidase in these low-glucuronidase strains is responsive to extrinsic factors. It is known that certain glucuronidogenic drugs will produce an increase in β-glucuronidase activity in the non-sex organs (Fishman, 1940)) and that estrogen will restore uterine β-glucuronidase activity of the castrated mouse to normal levels Fishman and Fishman, 1944; Fishman, 1947) with no alteration in the non-sex organ glucuronidase. (Fishman, 1947; Harris and Cohen, 1951). An exception to this lack of effect in the non-sex organs was reported by Kerr, Campbell, and Levvy (1949) who found that estrone but not estriol or estradiol produced an increase in liver β-glucuronidase. In all of these experiments, mice of mixed strain were employed. Accordingly, investigation was undertaken to explore the effect of extrinsic factors with regard to organ β-glucuronidase activity in inbred mice.

In a previous paper, (Fishman, 1951) it was reported that stilbestrol stimulated liver but not kidney β-glucuronidase activity in C3H mice whereas testosterone and methylandrostenediol produced a striking increase in kidney but not in liver β-glucuronidase. The present paper represents essentially a systematic investigation of the effects of the estrogen and androgen in one low-β-glucuronidase mouse strain (C3H) and in one

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high-β-glucuronidase strain (129). In addition, new data are presented on tissue glucuronidase in a number of mouse strains.

METHODS AND EXPERIMENTAL PROCEDURES

Mice of pure inbred strain were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine and, in general, were arranged in groups of 5 to 6 animals for the experiments. These mice, weighing 20 ± 3 grams, were approximately two months old.

β-Glucuronidase activity was determined by the method of Fishman, Springer, and Brunetti (1948) with modifications (Fishman, Kasdon, and Homburger, 1950) employing acetate buffer homogenates of tissue. These were prepared by homogenizing a small amount of tissue (15–60 mgm.) in 10 cc. of 0.1 ml acetate buffer (pH 4.5). Duplicate specimens of 0.1 cc. of the well-shaken homogenate were taken for analysis. Following the hydrolysis of phenolphthalein glucuronic acid, the liberated phenolphthalein was measured colorimetrically. β-Glucuronidase activity has been expressed in the usual units per gram of wet tissue. (Fishman, Springer, and Brunetti, 1948). In the case of seminal vesicles and uteri, the secretions were expressed and the tissues dried with filter paper. These tissues were then weighed on a microtorsion balance and later homogenized and assayed as described above.

Mice of both sexes of the following strains were sacrificed by cervical fracture and determinations were made of the β-glucuronidase activity of liver, kidney, and spleen; C3H, AKR, CAF_1, BAF_1, DAH and 129.

In order to study the effects of sex hormones on tissue β-glucuronidase, mice of both sexes of the C3H and 129 strains were selected and arranged in suitable groups. Untreated groups received injections of peanut oil (0.1 ml.) every other day for 14 days to serve as controls to the experimental groups which received 500 micrograms of either stilbestrol or testosterone propionate or both in 0.1 ml. of peanut oil under the same dosage regime. In the castrated animals, a period of approximately 14 days was allowed to elapse before injections were begun. In all mice, β-glucuronidase determinations were performed on the fresh liver, kidney, and spleen. Assays of uterine and seminal vesicle β-glucuronidase were done in female and male animals respectively.

Experiments were performed to discover the effect of testosterone propionate on kidney β-glucuronidase in mice of several pure inbred strains and to estimate a dosage-response curve for two strains of mice.

RESULTS

Strain and sex differences. Of the six strains studied, four (BAF_1, CAF_1, 129, DBA) were found to be high-B-glucuronidase strains and two (C3H, AKR) low-β-glucuronidase strains (Table 1). The Bar Harbor AKR can now be characterized as a low-β-glucuronidase strain as well as one highly susceptible to spontaneous leukemia. The kidneys of male mice of the BAF_1, CAF_1, AKR and DBA strains assayed higher glucuronidase activities than did the kidneys of female mice in the corresponding strain. However, mice of the C3H and 129 strains showed no sex difference with respect to kidney β-glucuronidase.

Effects of castration on tissue β-glucuronidase activity are recorded in Table 2 along with the significance of the differences of the means (Table 3).

TABLE 1. A STUDY OF TISSUE β-GLUCURONIDASE IN RELATION TO STRAIN AND SEX

Strain	Sex	Tissue β-glucuronidase activity (Units/Gram)		
		Kidney (No.)	Spleen (No.)	Liver (No.)
C_3H	F	309 (5)	1391 (5)	485 (5)
	M	238 (5)	1156 (6)	479 (6)
AKR	F	449 (12)	1938 (12)	415 (12)
	M	671 (18)	1748 (18)	519 (18)
BAF_1	F	1829 (6)	4940 (6)	4545 (5)
	M	2655 (6)	4632 (6)	3134 (6)
CAF_1	F	1803 (6)	3660 (6)	3425 (6)
	M	3780 (6)	3548 (6)	4045 (6)
129	F	1376 (6)	7712 (6)	3187 (6)
	M	1460 (6)	6878 (5)	4050 (6)
dba	F	1547 (8)	4515 (8)	3936 (8)
	M	3192 (10)	4613 (9)	4278 (10)

In the 129 strain, castration of the females resulted in an elevation of liver and spleen β-glucuronidase and a decrease in uterine enzyme, whereas in the male sex, only the seminal vesicles underwent a change (increase). In the females of the C3H strain, castration caused no change in β-glucuronidase activity except for a questionable increase in kidney enzyme. The male C3H mice behaved in a similar fashion to the 129 animals, only seminal vesicle β-glucuronidase increasing.

Testosterone propionate (Tables 2 and 3) produced marked elevations in kidney β-glucuronidase in mice of both sexes, intact and castrate, of the 129 and C3H strains. Other responses common to both strains were the reduction in uterine β-glucuronidase of the intact mice and the decrease in seminal vesicle β-glucuronidase. Whereas, in the C_3H mice, liver and spleen enzyme levels remained essentially unchanged, in the 129 mice, the liver of the intact female and liver and spleen of castrate males showed an increase while the spleen of castrate females exhibited a decrease in their enzyme values.

Stilbestrol administration (Tables 2 and 3). Except for the castrate male C3H mice, no experimental groups showed a change in kidney β-glucuronidase activity. Other findings common to both strains were the reduction in seminal vesicle β-glucuronidase in intact male mice and the increased activity in the castrated males. Although liver β-glucuronidase was elevated only in intact females of the 129 strain, this increase was a uniform finding in all four experimental groups of C3H mice. Increases in spleen β-glucuronidase were observed in both sexes of 129 strain mice but only in the males (both intact and castrated) of C3H mice. Uterine β-glucuroni-

TABLE 2. COMPARISON OF β-GLUCURONIDASE DATA IN C3H AND 129 STRAINS OF MICE

Exp. No.	Strain	Sex	Condition	Tissue β-glucuronidase (units/gram)				
				Kidney	Liver	Spleen	Uterus	Seminal vesicles
				A. (CASTRATION)				
1	129	F	Intact	1,376 (6)	3,187 (6)	7,712 (6)	2,827 (6)	
2	129	F	Castrate	1,536 (9)	4,566 (9)	11,401 (9)	1,498 (9)	
3	129	M	Intact	1,460 (6)	4,050 (6)	6,878 (5)		3,937 (6)
4	129	M	Castrate	1,404 (10)	4,258 (10)	6,136 (10)		15,070 (7)
5	C3H	F	Intact	301 (5)	481 (5)	1,391 (5)	1,036 (5)	
6	C3H	F	Castrate	403 (6)	575 (6)	1,536 (6)	1,085 (6)	
7	C3H	M	Intact	287 (10)	506 (10)	1,156 (6)		91 (6)
8	C3H	M	Castrate	302 (6)	651 (6)	1,536 (6)		3,230 (6)
				B. (TESTOSTERONE PROPIONATE)				
9	129	F	Intact	5,895 (6)	4,290 (6)	5,994 (6)	1,942 (6)	
10	129	F	Castrate	6,546 (5)	4,084 (5)	6,674 (5)	1,393 (5)	
11	129	M	Intact	6,098 (6)	3,400 (6)	6,487 (6)		3,400 (6)
12	129	M	Castrate	14,230 (5)	5,802 (5)	10,888 (5)		4,378 (5)
13	C3H	F	Intact	3,121 (6)	630 (5)	1,307 (5)	402 (6)	
14	C3H	F	Castrate	1,991 (6)	664 (6)	1,401 (6)	638 (6)	
15	C3H	M	Intact	2,738 (5)	503 (5)	1,394 (5)		139 (5)
16	C3H	M	Castrate	2,940 (4)	606 (4)	1,629 (4)		260 (4)
				C. (STILBESTROL)				
17	129	F	Intact	1,658 (6)	6,845 (6)	17,208 (6)	4,148 (6)	
18	129	F	Castrate	1,538 (5)	5,068 (4)	9,795 (4)	4,018 (4)	
19	129	M	Intact	1,504 (6)	4,557 (6)	13,238 (6)		8,856 (5)
20	129	M	Castrate	1,420 (5)	4,806 (5)	11,406 (5)		7,593 (4)
21	C3H	F	Intact	305 (6)	930 (6)	1,403 (6)	1,043 (6)	
22	C3H	F	Castrate	365 (6)	1,018 (6)	2,136 (5)	1,165 (6)	
23	C3H	M	Intact	364 (6)	986 (6)	1,866 (6)		1,704 (6)
24	C3H	M	Castrate	448 (6)	1,369 (6)	2,425 (6)		1,795 (6)
				D. (TESTOSTERONE PROPIONATE+STILBESTROL)				
25	129	F	Intact	2,355 (5)	6,558 (5)	10,444 (5)	3,572 (5)	
26	129	F	Castrate	1,991 (5)	5,892 (5)	8,852 (5)	3,378 (5)	
27	129	M	Intact	3,042 (4)	6,222 (4)	8,712 (4)		3,327 (4)
28	129	M	Castrate	1,283 (6)	3,870 (6)	8,113 (6)		26,250 (4)
29	C3H	F	Intact	1,062 (5)	1,153 (5)	1,413 (5)	801 (5)	
30	C3H	F	Castrate	1,051 (5)	1,052 (6)	2,381 (6)	1,058 (6)	
31	C3H	M	Intact	1,625 (6)	947 (6)	1,732 (6)		275 (6)
32	C3H	M	Castrate	1,902 (6)	1,176 (6)	2,378 (6)		238 (4)

TABLE 3. STATISTICAL SIGNIFICANCE OF DIFFERENCES OF MEAN VALUES RECORDED IN TABLE II; *t* AND P VALUES

Comparison of the means		Organ				
Exp. No.	vs. Exp. No.	Kidney	Liver	Spleen	Uterus	Seminal vesicles
			A. (CASTRATION)			
1	2		2.5(0.03)	3.7(0.01)	3.7(0.01)	
3	4					13.8(0.01)
5	6					
7	8					20(0.01)
			B. (TESTOSTERONE PROPIONATE)			
9	1	6.5(0.01)	5.2(0.01)		1.9(0.1)	
10	2	12.4(0.01)		4.5(0.01)		
11	3	21.2(0.01)				
12	4	20.0(0.01)	3.5(0.01)	6.1(0.01)		13.5(0.01)
13	5	20.0(0.01)			2.3(0.05)	
14	6	5.0(0.01)			3.3(0.01)	
15	7	10.4(0.01)				
16	8	3.8(0.01)				20(0.01)
			C. (STILBESTROL)			
17	1		6.5(0.01)	21.0(0.01)	2.5(0.04)	
18	2				3.2(0.01)	
19	3			2.5(0.03)		9.0(0.01)
20	4			4.1(0.01)		4.0(0.01)
21	5		4.0(0.01)			
22	6		8.4(0.01)			
23	7		8.0(0.01)	3.3(0.01)		20(0.01)
24	8	3.8(0.01)	5.4(0.01)	4.8(0.01)		3.1(0.01)
			D. (TESTOSTERONE PROPIONATE+STILBESTROL)			
25	9	4.5(0.01)	7.5(0.01)	5.1(0.01)	4.4(0.01)	
25	17	3.4(0.01)		3.9(0.01)		
26	10	8.8(0.01)	8.1(0.01)		7.8(0.01)	
26	18					
27	11	3.2(0.01)	8.1(0.01)			
27	19			2.2(0.06)		
28	12	23.1(0.01)	6.8(0.01)			+
28	20					+
29	13	2.8(0.02)	5.4(0.01)		6.6(0.01)	
29	21	4.8(0.01)				
30	14	2.9(0.02)	5.1(0.01)	5.1(0.01)	6.8(0.01)	3.5(0.01)
30	22	15.0(0.01)				
31	15	2.8(0:02)	4.4(0.01)			4.2(0.01)
31	23	4.2(0.01)				14.8(0.01)
32	16	1.3(0.2)	4.7(0.01)	6.4(0.01)		
32	24	4.6(0.01)				13.2(0.01)

Statistical studies were done only where the differences between the means were greater than 33% of the lesser value. This accounts for the blank spaces in the table.

dase was raised in 129 intact and castrate females but was not changed in the corresponding groups of the C3H strain.

Testosterone propionate plus stilbestrol (Tables 2 and 3) Androgen and estrogen exhibited neutralizing actions on each other in some organs but not in others depending on the strain, the sex and the presence or absence of the gonads. Thus, estrogen, sometimes partially and on other occasions wholly, prevented the characteristic elevation of kidney β-glucuronidase by testosterone propionate in both strains. However, the androgen did not prevent (except for experiment 28) the estrogen-induced increases in liver β-glucuronidase of C3H (experiments 29, 30, 31, 32) and 129 (experiment 25) mice or affect the liver enzyme values (experiments 26, 27 and 28). In the spleen, androgen counteracted the enzyme-enhancing effect of estrogen only in intact male and female 129 mice, while, in the uterus, androgen effected little or no change in the β-glucuronidase activity when compared with the values seen in the groups given estrogen alone. Seminal vesicle β-glucuronidase maintained levels similar to those of corresponding groups given androgen alone in all experiments except number 28.

Dosage-response curves. Data relating kidney β-glucuronidase to dosage of testosterone propionate in C3H and A Cloudman male mice appear in Figure 1. Evidently, testosterone propionate in physiological amounts is capable of causing a significant response in kidney β-glucuronidase.

Kidney glucuronidase response to testosterone in various strains of mice. The kidney β-glucuronidase response to testosterone propionate differs with the strain of animal employed (Table 4). Thus, two levels of stimulation were observed. The 129 and DBA strains exhibit a 4 to 5-fold increase

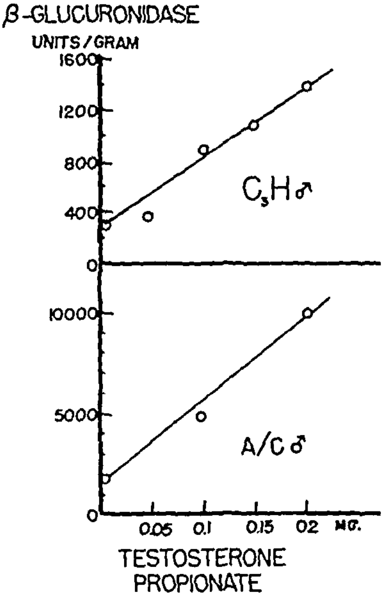

FIG. 1. Testosterone propionate dose-response curve. Each experimental point is the average of the β-glucuronidase activities of the kidneys of each group of 6 male mice. The daily dosage of testosterone propionate is indicated in the figure and the length of the experiment was 14 days.

and the C3H, AKR, CAF_1, BAF_1, and A Cloudman, a 9.5 to 12.5-fold elevation. It may be noteworthy that both low-β-glucuronidase strains fall into the second category.

TABLE 4. KIDNEY β-GLUCURONIDASE RESPONSE TO TESTOSTERONE PROPIONATE IN INBRED MICE (MALE)

Strain	Control animals	Experimental animals	Percentage increase
129	1,460 (6)	6,098 (6)	416
C3H	287 (10)	2,738 (5)	955
AKR	650 (6)	5,875 (6)	1,110
DBA	2,821 (10)	14,214 (7)	505
A Cloudman	3,396 (6)	42,075 (4)	1,245
BAF_1	2,655 (6)	26,300 (7)	990
CAF_1	3,780 (6)	25,450 (7)	675

DISCUSSION

In this study a new low β-glucuronidase strain (Bar Harbor AKR) has been discovered. At present, the following strains are known to belong to this category, C3H (Heston, Bittner, Andervont) DBA (Andervont). Law, Morrow and Greenspan (1952) have reported that the inheritance of the low liver β-glucuronidase in C3H mice is a characteristic of a single recessive gene. Although strain differences have been reported for a few other enzymes, this phenomenon has been studied systematically only with regard to the inheritance of β-glucuronidase. It would appear, therefore, that in mice, the genetic background of the animal is an important factor in determining tissue β-glucuronidase activity.

It has now been established, confirming our previous data (Fishman, 1951) that sex hormones have the ability of increasing the β-glucuronidase activity of certain tissues in mice of both a low- and a high-β-glucuronidase strain.

Androgen

In the case of androgen (testosterone propionate), it was possible to increase regularly the kidney β-glucuronidase to levels far above the highest values encountered in any untreated normal mice. This phenomenon has been demonstrated in both C3H and 129 strains of mice (Table 2) and in five other strains studied. In the 129 strain, on several occasions, β-glucuronidase elevations were seen also in liver and spleen, such changes being absent in corresponding groups of C_3H mice. Testosterone propionate prevented the characteristic rise in β-glucuronidase of the seminal vesicles which ordinarily follows the castration of males of both strains.

Estrogen

The action of estrogen (stilbestrol) on the other hand, differed from androgen in that kidney β-glucuronidase was essentially unaffected, and liver and spleen β-glucuronidase showed significant increases, the extent of which depended on the strain and condition of the animals. However, in no experiment in C3H mice was it possible to increase liver β-glucuronidase to the levels found in high β-glucuronidase strains. This finding contrasts with the behavior of kidney β-glucuronidase in testosterone-treated low-glucuronidase strains. Seminal vesicle β-glucuronidase was increased by stilbestrol. The experiments in Table 2 were all repeated using 5 γ instead of 500 γ of estrogen and the corresponding results were essentially identical except that, in the 129 strain, spleen β-glucuronidase was in the range of the corresponding untreated control animals and the liver β-glucuronidase of C_3H mice did noot increase.

Testosterone-propionate and stilbestrol

In these experiments, stilbestrol always counteracted the action of androgen on kidney β-glucuronidase but androgen had no antagonistic action towards stilbestrol's action on liver, and only occasionally, on spleen β-glucuronidase. The data dealing with the uterus show a domination of the estrogen effects and those concerning seminal vesicles, except for experiment 28, resemble closely the results with androgen alone.

Another point of interest is the following strain difference: the antagonistic effect of estrogen on androgen-increased kidney β-glucuronidase is more marked in the 129 mice than it is in the C3H strain.

It appears that kidney β-glucuronidase is more specifically influenced by androgen and estrogen than any of the other tissues studied. It is also clear from other unpublished experiments dealing with borneol administration that the increase found in liver β-glucuronidase is by no means specific for stilbestrol. The possibility noted here of strain and sex differences in the ability of the liver to increase its β-glucuronidase activity with estrogen stimulation may explain the discrepancy in the reports of Kerr, Campbell and Levvy (1949) and Fishman (1947).

Castration effects

Clear-cut strain and sex differences have been demonstrated in the experiments concerned with the castration of 129 and C3H mice. Thus, castrated females of the 129 strain exhibit significant increases in liver and spleen β-glucuronidase which are not seen in the corresponding experiments with C3H mice; castrated males of the same strain do not show this increase. Castrated females of 129 mice (as has been reported for White Swiss animals (Fishman and Fishman, 1944; Fishman, 1947) show a significant decrease in uterine β-glucuronidase which is not seen in C3H castrate female mice. On the other hand, castrate male mice of both strains show a marked rise in the β-glucuronidase activity of the seminal vesicles.

Dosage-response curves

As seen from the dosage-response curves, significant elevations in kidney β-glucuronidase activity were obtained with amounts of testosterone propionate as little as 0.1 mg. per day. The maintenance of the weight of the seminal vesicles has required as much as 1 mg. per day. Accordingly, on the basis of these minimal effective doses, it may be stated that the kidney β-glucuronidase response to androgen is a physiological phenomenon.

Enzyme response to androgen in various strains

The comparison of the kidney β-glucuronidase response to testosterone propionate in inbred mouse strains indicates real quantitative differences ranging from 4 to 13-fold increments. As the only variable which is evident is the strain of the animal in these experiments, it is reasonable to assume that the β-glucuronidase response to testosterone is strain-dependent. If it is correct to assume that this enzyme phenomenon is an important aspect of androgen action in the kidney, the fundamental question follows as to whether or not sex hormone effects in general are influenced to an important degree by heredity.

CONCLUSIONS

1. A new low-β-glucuronidase mouse strain (AKR) has been found; liver, kidney and spleen exhibiting enzyme activity in the range observed for previously reported low-β-glucuronidase strains of inbred mice.
2. A comparison was made in one high- (129) and in one low- (C_3H) β-glucuronidase strain of the effects on tissue β-glucuronidase of castration and the administration of stilbestrol and testosterone propionate, alone and in combination.
3. Testosterone propionate produced marked elevations in kidney β-glucuronidase in mice of both sexes, intact and castrate, of the 129 and C3H strains. This action was neutralized usually in part and sometimes completely by simultaneously administered stilbestrol.
4. Stilbestrol increased the activity of liver β-glucuronidase in C3H mice, although to a much lower level than was characteristic of the liver values in the high-glucuronidase strains.
5. Following castration of male mice of 129 and C3H strains, seminal vesicle β-glucuronidase increased. Testosterone propionate neutralized this action.
6. From dosage-response curves completed in two strains of mice (C3H, A Cloudman), it appears that kidney β-glucuronidase responded significantly to 1.4 mg. of testosterone propionate given in divided doses over 14 days.

7. The response of kidney β-glucuronidase to testosterone propionate differed with the strain of mice employed.

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EXTENDED SURVIVAL OF ADRENALECTOMIZED RATS POSSESSING EAR- OR SPLEEN-GRAFTED OVARIES[1,2]

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IN MOST mammals removal of the adrenal glands results in deleterious changes leading to loss of body weight and terminating in death after a variable, usually brief, period of time. The female ground squirrel (*Citellus tridecemlineatus*) is apparently an exception to this rule since it lives for periods up to one year following adrenalectomy (Groat, 1943). Its ovaries are altered to the extent that some ovarian cells are described as "adrenocortical-like" and it is this altered histology that is held to be accountable for increased survival.

If an ovary is grafted to the ear of a castrate mouse or rat it becomes morphologically and functionally different from control ovaries in that it possesses thecal luteinization and it secretes an androgen (Hill, 1937a, 1937b; Deanesly, 1938). An ovary transplanted to the spleen of a rat becomes markedly luteinized, even to the point of luteoma formation, after a period of time (Biskind and Biskind, 1944). These two technics for producing deviations from normal ovarian structure and function have been used to study the possibility that transplanted ovaries, like the intact ones of the ground squirrel, may protect adrenalectomized animals against death from adrenal insufficiency. Hill (1948, 1949) demonstrated that ovaries grafted to the ears or spleen of mice can extend survival time following adrenalectomy (averages, 121–137 days as compared to 6.7–12.0 days for adrenalectomized controls). The present experiment indicates that grafted ovaries can extend survival following adrenalectomy in the albino rat as well and that ovaries grafted when immature are as effective as mature ovaries.

MATERIALS AND METHODS

200 male and female albino rats of the Wistar strain were used in this experiment. 189 of these were divided into four groups:

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[2] Portions of this material were presented in theses in partial fulfillment of the requirements for the degree of Master of Science (I.J.L. and M.H.G.).

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Group I. Adrenalectomized controls. 16 mature males and 40 mature females (weight 38–170 gm.) were subjected to no operative procedures before adrenalectomy.

Group II. Sham-operated adrenalectomized controls. 20 males and 17 females at the age of 21 days (weight 20–42 gm.) were castrated and implanted with a piece of uterus the size of an immature ovary into the spleen.

Group III. Ear-implanted experimentals. 29 mature females (weight 40–170 gm.) were castrated and autoplastically implanted with ovaries into the ears.

Group IV. Spleen-implanted experimentals. 27 males and 29 females at the age of 21 days (weight 19–46 gm.) and 11 mature females (40–170 gm.) were castrated. The females were then autoplastically implanted with an ovary into the spleen and the males were implanted with an ovary from a littermate female into the spleen.

All animals of all groups were bilaterally adrenalectomized 2–4 weeks after previous operations or when they matured. All animals were maintained on Purina laboratory chow and tap water, both *ad libitum*. The rats were weighed every 4 days following adrenalectomy, and they were examined daily to determine survival. The body weights and survival times in days following adrenalectomy were recorded for rats found dead. Since most control rats died within 25 days we arbitrarily considered as survivors only those rats that lived at least 3 times that long (75 days). Rats surviving 75 days or more were examined for presence of grossly visible adrenal regrowths and rests in the abdominal cavity, especially in the area of the kidneys. Reproductive function of 5 ear-implanted and 3 spleen-implanted mature females which had survived 168 days after adrenalectomy was studied by observing vaginal smears and copulation plugs. Standard histological technics were employed to study the structure of the grafts and suspected adrenal regrowths and rests.

Those experimental rats surviving more than 75 days were subjected to a standardized stress test (Marmorston-Gottesman and Gottesman, 1928) consisting of intraperitoneal injections of 15 mg. per 100 gm. body weight of histamine diphosphate. Under the conditions of this test intact rats survive and completely adrenalectomized rats die within 24 hours. Six intact and 5 newly adrenalectomized (3–4 days postoperative) rats were used as controls. By the use of the test it was hoped that the survival of rats possessing ovarian grafts could be compared with that of intact and adrenalectomized controls for the purpose of determining the adrenocortical-like protective action of the ovarian grafts. The time of death was noted for those rats which died within 24 hours after the histamine injection. Death within 24 hours was considered evidence for the presence of adrenocortical insufficiency.

RESULTS

Survival. The results regarding survival are summarized in Table 1. Group I (adrenalectomized controls). 8 out of 56 rats which lived longer than 75 days (range, 102–168 days) were found to possess large adrenal regrowths and hence were eliminated from consideration. 45 out of the remaining 48 adrenalectomized rats died within 23 days after adrenalectomy (mean, 13.7 ± 0.6 days; range, 4–23 days) and one died on the 53rd day. Two rats of this group survived 88 and 95 days; no adrenal tissue could be found to account for their prolonged survival. As tested by the χ^2 method, no significant differences in survival were found between males and females (see Table 2).

TABLE 1. SUMMARY OF SURVIVAL DATA

Group	No. of rats	No. eliminated for regrowths	No. considered	No. surviving 75 days or more	No. surviving 30 days or less
Adrenalectomized controls	56	8	48	2	45[1]
Sham-operated adrenalectomized controls	37	2	35	3	32
Ear-implanted experimentals	21	1	20	4	16
Spleen-implanted experimentals	67	2	65	18	42[2]

[1] One rat died 53 days after adrenalectomy.

[2] Five rats died 35, 50, 53, 55, and 56 days after adrenalectomy.

Group II (sham-operated adrenalectomized controls). 32 out of 37 adrenalectomized rats each bearing a uterine graft in the spleen died within 18 days after adrenalectomy (mean, 11.3 ± 0.5 days; range, 5–18 days). Two rats were eliminated because of adrenal regrowths. The remaining 3 rats of this group survived 76, 78, and 113 days after adrenalectomy and were then killed for autopsy. No significant differences in survival were found to exist between males and females of this group.

Since the χ^2 test revealed no significant difference in survival between rats of Groups I and II, the results of both control groups may be combined: 5 out of 83 combined controls survived 75 days following bilateral adrenalectomy.

Group III (ear-implanted experimentals). 4 out of 21 rats of this group had survived up to the time they were killed on the 168th day after adrenalectomy. A 5th survivor possessed a large adrenal regrowth and was eliminated from the results. The other 16 rats died within 30 days (mean, 13.6 ± 1.5 days; range, 7–29 days). We shall define a survival ratio (a/d) as the ratio of the number of rats which survive at least 75 days after adrenalectomy, a, to those which survive less than 30 days, d. For this group it is 4/16, which is significantly greater than that of the combined controls (5/77).

TABLE 2. STATISTICAL ANALYSIS OF SURVIVAL DATA

Hypothesis	X^2	p
Survival ratio* is independent of:		
1. Sex of adrenalectomized controls	.979	$>.30$
2. Sex of sham-operated adrenalectomized controls	.580	$>.30$
3. Sex of immature spleen-implanted experimentals	.164	$>.50$
4. Site of graft: ear or spleen	.752	$>.30$
5. Presence of uterine (sham) grafts in adrenalectomized controls	.653	$>.30$
6. Presence of ovarian grafts	13.355	$<.01$**

* Survival ratio is the ratio of the number of rats surviving 75 days or longer to those surviving less than 30 days after adrenalectomy.

** Hypothesis is rejected at the 1% level of confidence. The 5 preceding hypotheses are accepted.

Group IV (spleen-implanted experimentals). 2 out of 67 rats possessing ovarian implants in the spleen survived more than 75 days after adrenalectomy but were eliminated from consideration because they possessed large adrenal regrowths. 18 of the remaining 65 spleen-implanted rats survived at least 75 days after adrenalectomy (maximum, 168 days) before being killed and were found to possess no adrenal rests or regrowths. 42 of the remaining 47 rats died within 30 days (mean, 14.7±0.7 days; range, 8–30 days) and 5 rats of this group died 35, 50, 53, 55, and 56 days following adrenalectomy. No significant survival differences were found between males and females, or between rats with mature and immature ovarian grafts. The ratio (a/d) of the number of rats which survived at least 75 days after adrenalectomy, a, to those which survived less than 30 days, d, is significantly higher for this experimental group, 18/42, than for the controls, 5/77.

Statistical analysis shows that no significant survival difference exists between the rats with ovaries grafted to the ears and those with ovarian grafts to the spleen. Thus the combined survival ratio for groups III and IV is 22/58.

Body weight. The body weights of all rats which survived 30 days or less showed similar rates of decline. Death from adrenocortical insufficiency was consistently preceded by a precipitous drop in body weight. In general those rats which survived 75 days or longer showed parallel weight gains regardless of whether they had received ovarian or uterine grafts or none at all.

Adrenal regrowths and rests. Thirteen rats which had survived 75 days were found to possess gross adrenal regrowths and had to be eliminated from the survival results (see above). These regrowths appeared at the usual site of the adrenal and sometimes reached normal adrenal size. Histological examination of the regrowths showed that cords of adrenocortical cells differentiated from remnants of the adrenal capsule. In no cases were adrenocortical rests as such found in any rats.

Ovarian function. The 4 mature female ear-grafted rats which survived adrenalectomy were examined for evidence of estrous cycles and mating behavior. Vaginal smears over a 20-day period showed that all of the rats with ovaries grafted to the ears showed normal 4–5 day estrous cycles. When in estrus these rats were observed to mate readily with normal males. Presence of copulation plugs confirmed that mating had occurred. The rats with ovaries grafted to the spleen showed no evidence of estrous cycles, nor would they mate.

Histology. Only spleen-grafted ovaries were examined in sufficient numbers to permit adequate histological analysis. Ovarian grafts from animals which survived for a significant period of time after adrenalectomy could not consistently be distinguished from grafts taken from rats which died within the control period. In general, the histology of these ovaries was similar to that of the grafted ovaries described by Biskind and Biskind (1944). Ovaries from both groups varied in size from 2 to 10 mm. in diameter and some had follicles in all stages of development, with and without ova, and some follicles were cystic. All ovarian grafts were heavily luteinized or had infiltration of connective tissue into the corpora lutea. The ovarian grafts from animals that failed to survive usually were organized into discrete, normal-appearing corpora lutea, often surrounded by scanty connective tissue stroma. On the other hand the ovaries of rats which survived beyond the control period showed evidence of thecal luteinization and possessed islands of luteal tissue not organized into corpora lutea. These ovaries often appeared to be disorganized and contained relatively generous amounts of connective tissue stroma. The corpora lutea of these ovaries were rarely normal in appearance due to presence of non-luteal cells interspersed among luteal cells, replacement of the centers by connective tissue, or complete absence of typical luteal cells. In no case did rats possessing ovaries without these histological features survive beyond the control period.

TABLE 3. RESULTS OF THE HISTAMINE TEST FOR ADRENOCORTICAL FUNCTION

Group	No. of rats	No. of rats surviving <24 hrs. after histamine	No. of rats surviving >24 hrs. after histamine
Intact rats	6	0	6
Newly adrenalectomized rats (3–4 days before histamine injection)	5	5	0
Adrenalectomized controls	3	0	3
Sham-operated adrenalectomized controls	3	3	0
Ear-implanted experimentals	4	0	4
Spleen-implanted experimentals	14	9	5

Histamine tests. Table 3 summarizes the results of the histamine tests. Three male and 2 female rats adrenalectomized 3–4 days before injection of histamine became prostrate and succumbed within an hour after injection. Three intact males and 3 intact females all regained usual activity within 2 hours and survived an observation period of 24 hours. The ability of spleen-grafted ovaries to prolong survival times of adrenalectomized rats did not necessarily equip them to withstand histamine stress. This is seen in the fact that 9 out of 14 of the rats which received ovarian grafts to the spleen failed to survive 24 hours after injection.

DISCUSSION

The data presented here indicate that rat ovaries, transplanted to the ears or spleen of albino rats, protect a significant proportion of such rats against weight loss and death from adrenocortical insufficiency for 75 days or longer following adrenalectomy. These results corroborate those of Hill (1948) who observed that out of a total of 105 mice with ovaries grafted to the ears, 52 survived beyond the 29 day limit for adrenalectomized controls. In the present experiment 20% of the ear-implanted rats survived more than 75 days after adrenalectomy, whereas survival was 49.1% for Hill's ear-implanted mice. In mice with ovaries grafted to the spleen, Hill (1949) found that 32 out of 47 females, or 68.1%, survived variable periods beyond 30 days. Survival beyond 75 days following adrenalectomy for our spleen-implanted rats was 27.7% compared to 68.1% survival beyond 30 days for Hill's spleen-implanted mice. It is difficult to explain these species differences; however it should be emphasized that these figures represent more agreement than they seem to show since Hill did not apply the criterion that animals must live at least 75 days after adrenalectomy in order to be counted as survivors.

The survival-prolonging function of rat ovaries is apparently independent of other ovarian functions. Since ovaries of immature rats afford protection against death from adrenocortical insufficiency equal to that of ovaries of adults, the present data eliminate maturation and estrous cycling of ovaries previous to transplantation as a necessary condition for prolongation of survival time.

Deanesly (1938) described a process of "thecal luteinization" in ovaries grafted to the ears of castrated male rats. She attributed the observed androgenic activity of such ovaries to their state of thecal luteinization. In the present experiment, ovaries in rats whose survival exceeded 75 days after adrenalectomy possessed extensive luteinization beyond the limits of any corpus luteum. Many of the rats which died within the control period had ovaries with a large amount of luteal tissue but in all cases it was confined to the limits of corpora lutea. It is likely that this difference in organization of the luteal tissue in the two groups is associated with differences in survival. Very possibly the cells of the ground squirrel ovary which Groat (1943) described as "adrenocortical-like" were, in fact, luteal cells.

It is not understood why survival was not extended for all adrenalectomized mice and rats possessing ovarian transplants. However not too much should be expected of grafted ovaries since even adrenals transplanted to similar sites offer only partial effectiveness with respect to life-prolongation (Stolpe and Schreiber, 1952).

Komrad and Wyman (1950) found that adrenal glands auto-transplanted to the dorsal musculature could protect all their rats against injection of histamine (in the same form and dosage as in the present experiment) after a minimum of 7 days following transplantation. However, more than half of our rats with spleen-grafted ovaries did not survive injection of histamine, although all 4 rats with ear-grafted ovaries did survive. Stolpe and Schreiber (1952) found that when adrenal glands were auto-transplanted to the same sites, spleen or ears, recipient rats survived histamine stress no better than rats with ovarian transplants unless sufficient exogenous ACTH was previously administered. It may be possible that grafted ovaries require stimulation by an anterior pituitary hormone in order to exercise full protective action against histamine stress.

Two hypotheses suggest themselves in explanation of the mechanism of the life-prolonging action of grafted ovaries. The first is that the grafted ovaries, in consequence of their ectopic position, secrete one or more substances qualitatively different from other ovarian secretions and life-prolonging in action. A precedent for this supposition is the androgenic function of ear-grafted ovaries in mice and rats (Hill, 1937a, 1937b; Deanesly, 1938; Lampton and Miller, 1940; Hernandez, 1943).

The second hypothesis is that the grafted ovaries, so highly luteinized, secrete sufficient progesterone to account for increased survival after adrenalectomy. Much evidence, direct and indirect, has indicated that progesterone is able to increase survival of rats for rather short periods of time following adrenalectomy (Gaunt, Nelson and Loomis, 1938; Greene, Wells and Ivy, 1939; Emery and Greco, 1940). However, experiments in our laboratory demonstrate that progesterone is ineffective in prolonging survival of rats more than 2 months after complete removal of adrenocortical tissue. For this reason, the first hypothesis is preferred.

SUMMARY

Twenty adult female Wistar rats were castrated and autoplastically implanted with an ovary into each ear. Eleven adult females were castrated and autoplastically implanted with an ovary into the spleen. Fifty-four 21-day old males and females were also castrated and auto- or homoplastically implanted with an immature ovary into the spleen. These rats were adrenalectomized 2 to 4 weeks postoperatively, or when mature. Forty-eight adult and immature adrenalectomized rats and 35 immature castrated sham-implanted adrenalectomized rats served as controls.

Seventy-seven out of 83 control rats died within 30 days after adrenalectomy, and 5 survived 75 days or longer. Four out of 20 ear-implanted and 18 out of 65 spleen-implanted experimental rats survived 75 days or longer,

a significantly larger proportion than among the controls. There were no differences in survival ratio between the sexes, between mature and immature rats, or between ear- and spleen-implanted rats.

Ovarian grafts from surviving rats possessed abundant luteal tissue, much of it not organized within corpora lutea, whereas the ovarian grafts from animals which died within the control period had luteal tissue mostly restricted to corpora lutea.

The ear-grafted ovaries supported normal ovarian cycling and mating behavior and protected against death from histamine poisoning. The spleen-grafted ovaries exhibited no cyclic behavior and protected only 9 out of 14 surviving rats against death from histamine poisoning.

It is hypothesized that ear- and spleen-grafted ovaries, in consequence of their ectopic position, secrete one or more unknown substances which have a life-prolonging action for adrenalectomized rats.

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THE EFFECT OF GROWTH HORMONE ON TIBIA GLYCOGEN CONTENT OF THE HYPOPHYSECTOMIZED RAT[1]

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GLYCOGEN has been implicated in endochondral calcification by Gutman and Yü, (1950) in their studies on the effect of several specific inhibitors on the enzymes of the glycolytic system. Moreover, Marks and Shorr (1950) have shown that a marked diminution of the *in vitro* calcification of cartilage slices occurs after a preliminary amolytic digestion of the glycogen present in epiphyseal cartilage. Since in the immature hypophysectomized rat ossification is markedly retarded, it was considered of interest to determine the effect of hypophysectomy on the glycogen content of actively growing bone. Furthermore, since the administration of growth hormone reactivates osteogenesis in immature hypophysectomized rats, a study was made of the effects of growth hormone treatment on the glycogen content of bone.

METHODS

Female rats of the Long-Evans strain were used. Normal animals twenty-one days and older had unrestricted access to Diet XIV; hypophysectomized animals were given Diet XIV and lettuce, supplemented with wet Diet I.[2]

For the determination of glycogen, the following procedure was employed. The left tibia of the rat was removed under amytal anaesthesia and rapidly cleaned of muscle and connective tissues. The fibula was cut off flush with the surface of tibia and discarded. After cutting the tibia in half both length and cross wise, the tibia was placed in 2 ml. hot 30% KOH and digested for 2 hours in a boiling water bath. The centrifuged digest was then transferred into a test tube graduated at 3 ml. volume and the bone residue remaining was washed twice with ½ ml. portions of 30% KOH. To the 3 ml. volume of the digest, 0.3 ml. 5% $NaSO_4$ was added followed by 4 ml. absolute ethanol. After mixing and bringing to a momentary boil, the tubes were chilled for at least 2 hours at 5° C. The precipitate was then centrifuged and the supernatant fluid decanted.

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[2] Diet XIV consists of ground whole wheat 67.5%, casein 5%, alfalfa leaf meal 10%, fish meal 10%, fish oil 5%, NaCl 1.5%. Diet I consists of ground whole wheat 67.5%, casein 15%, skim milk powder, 7.5%, NaCl 0.75%, $CaCO_3$ 1.5%, melted fat 6.75%, fish oil 1.0%.

The precipitate was washed once with 70% and then 95% ethanol and dried. After dissolving the precipitate in either 3 or 5 ml. of water, depending on the quantity of glycogen expected, 0.8 ml. aliquots were removed for analysis by a modification of the anthrone method (Viles and Silvermann, 1949). The 0.8 ml. aliquots were delivered into 3 ml. glass stoppered volumetric flasks containing magnetically stirred "fleas." The flasks were then chilled in a crushed ice bath. With the "flea" stirring vigorously, 2.0 ml. of anthrone reagent (0.15% in 95% H_2SO_4) was added rapidly and stirred exactly one minute. The volumetric flasks were then transferred to a boiling water bath for exactly 7 minutes at which point maximum color development occurs under the conditions here employed. The flasks were then chilled rapidly in the crushed ice bath and diluted to volume with 2.5:1 H_2SO_4:H_2O. Optical density was measured at 625 mμ. Standards of twice reprecipitated glycogen were analyzed with each series of samples, since color development varies slightly with the age of the anthrone reagent. Analyses of known solutions of glycogen average 98% recovery by this method.

Solutions of purified chondroitin sulfate react with anthrone reagent to give an absorption curve with a maximum at 625μ. The presence of chondroitin sulfate in cartilage presented the possibility of interference in the determination of glycogen. This was ruled out by digestion of purified solutions of chondroitin sulfate in 30% KOH for 2 hours at boiling water bath temperature, after which time virtually no color development occurs. Furthermore, digestion of the glycogen obtained from bone with salivary amylase and analysis according to the above procedure resulted in complete absence of color development indicating that the chromogenic substance being determined is glycogen uncontaminated with chondroitin sulfate.

The growth hormone used was prepared according to the procedure of Wilhelmi *et al.* (1948) from beef anterior pituitaries and was electrophoretically homogeneous at pH 4.0 and 10.0. The minimum effective doses for the demonstration of contaminating hormones were determined histologically on 26 day old hypophysectomized female rats (injected for 4 days) and were found to be as follows: thyrotropic, 0.5 mg.; interstitial cell stimulating, 0.5 mg.; adrenocorticotropic, >2 mg.; follicle stimulating, >2 mg. The minimum effective dose for growth hormone was 10 gamma as determined by the tibia assay method of Greenspan *et al.* (1949).

RESULTS

Variations in Tibia Glycogen Content with Age in Normal Animals and with Post-operative Period in Hypophysectomized Animals. The tibias of groups of normal female rats varying in age from 12 to 50 days were analyzed for glycogen. The analytical values are presented in Figure 1 in graphical form and are plotted as a function of age. It is evident from the curve that a maximum glycogen content in the tibia is reached at approximately 20 days of age following which there occurs a rapid decline particularly notable between 20 and 26 days and declining less precipitously thereafter. In the period between about 26 and 45 days the glycogen content of the tibia remains quite constant but appears to drop sharply after this age.

Female rats after hypophysectomy at 26 days of age contain considerably less glycogen in their tibias compared to normal animals of corresponding age. The quantitative values are presented in the curve for hypophysectomized animals in Figure 1. Quite outstanding is the marked decrease in glycogen content of the tibia which occurs during the two weeks following hypophysectomy. Thereafter the decline ceases and a constant glycogen content appears to be maintained.

Effect of Extended Treatment with Growth Hormone on Glycogen Content of Tibia of Hypophysectomized Rats. The animals constituting this series of experiments were injected with 150 gamma of the purified growth hormone daily beginning with the day following hypophysectomy. One group of animals was thus injected for 7 days, a second for 14, and a third group for 21 days. The results of the various periods of growth hormone treatment are given in Figure 1 (Hyp+G.H.). It is evident that treatment of hypophysectomized female rats under the given conditions maintains the glycogen content of the tibia at a normal level during 1 and 2 weeks of injection with growth hormone following hypophysectomy. Furthermore, whereas the glycogen begins to fall rather sharply after 45 days of age in normal animals, the glycogen content of the treated hypophysectomized

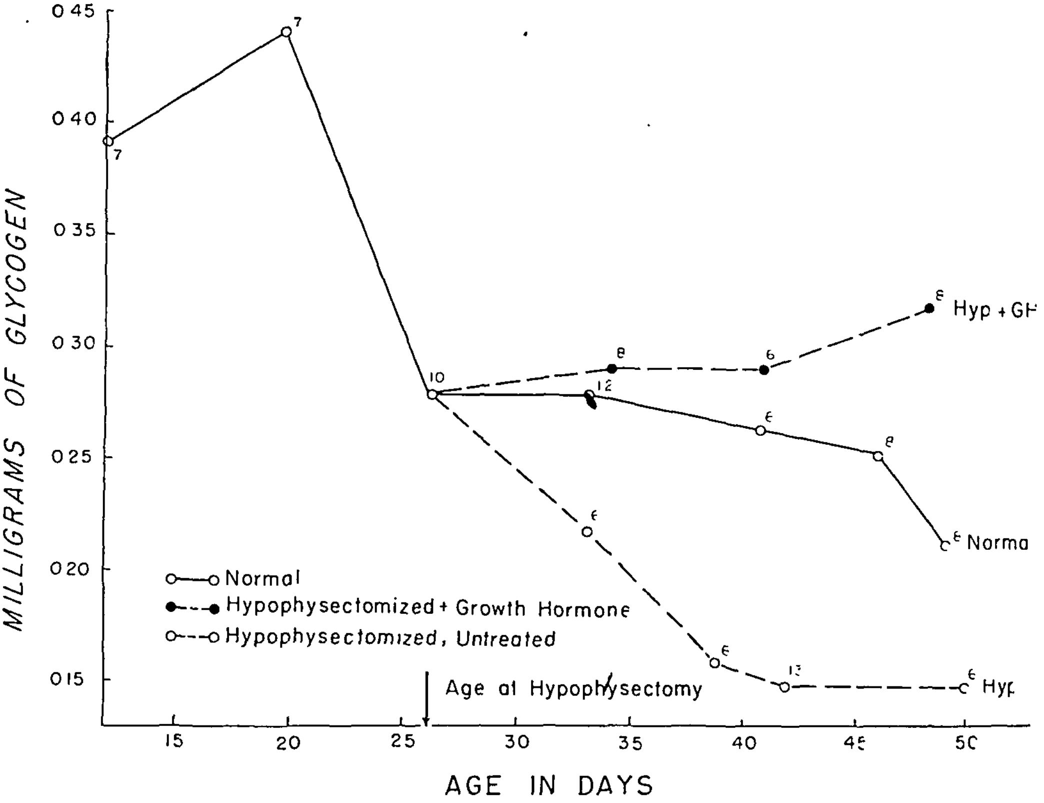

FIG. 1. The variation of tibia glycogen content with age in normal rats, with postoperative period in hypophysectomized rats, and the effect of growth hormone treatment of hypophysectomized rats. The figure adjacent to each point represents the number of tibias analyzed.

rat tibia of the corresponding age is still increasing after 3 weeks of injection.

Effect of Growth Hormone on Tibia Glycogen Content of Two-week Postoperative Animals. The data presented in the preceding paragraphs indicates that growth hormone is capable of preventing the marked decline in the tibia glycogen content which follows hypophysectomy. In order to determine whether growth hormone is capable of restoring to normal the glycogen content of tibia which are already depleted of glycogen, the following experiment was performed. Hypophysectomized female rats 39 days old, 13 days postoperative were injected daily for 4 days with various doses of growth hormone. The animals were autopsied on the fifth day and tibia glycogen analyzed. The results are presented in Table 1 and indicate that the glycogen content can be restored to normal if sufficiently large doses are given.

TABLE 1. THE EFFECT OF GROWTH HORMONE ON TIBIA GLYCOGEN OF HYPOPHYSECTOMIZED RATS

Number of animals	Type and age	Total dose growth hormone per 4 days (mg.)	Tibia glycogen (mg.)[1]
6	Normal—41 days old	0	0.26 (0.23–0.29)
6	Hypophysectomized 26 day +13 day postoperative	0	0.15 (0.13–0.16)
5	Hypophysectomized 26 day +13 day postoperative	0.1	0.17 (0.16–0.19)
6	Hypophysectomized 26 day +13 day postoperative	0.5	0.20 (0.19–0.23)
6	Hypophysectomized 26 day +13 day postoperative	1.0	0.24 (0.20–0.27)

[1] Mean value and range of variation.

Normal female rats, 50 days old and averaging 110 grams weight, when treated with a 2 mg. dose of growth hormone for 4 days showed no change in tibial glycogen compared with uninjected controls. Over this period of treatment, the injected animals averaged a 5 gram weight gain above the controls.

Effect of Growth Hormone on Tibia Glycogen Content of Fasted Hypophysectomized Rats. In fasting hypophysectomized rats, glycogen stores in the gastrocnemius and heart muscle are abnormally depleted (Illingworth and Russell, 1951). However, when growth hormone is administered to such animals during the fast, restoration of glycogen stores to fasting levels of the intact animal occurs. To determine whether tibia glycogen behaves similarly the following experiment was carried out. Hypophysectomized female rats were fasted 24 hours receiving growth hormone at 0 and 12 hours of fasting. The glycogen content of the tibias as compared to that at onset of fasting and in uninjected fasting hypophysectomized controls at time of autopsy are presented in Table 2. The data indicate that neither fasting nor growth hormone treatment effected any alteration in the glycogen content of the tibia. The ineffectiveness of growth hormone under these conditions is probably attributable to the relatively short duration of treatment. However, the 24 hour period of fasting is adequate to deplete muscle glycogen stores abnormally in hypophysectomized rats (Russell, 1950).

TABLE 2. THE LACK OF EFFECT OF GROWTH HORMONE ON TIBIA GLYCOGEN OF FASTING HYPOPHYSECTOMIZED RATS

Number of animals	Type and age	Treatment	Tibia glycogen[1] (mg.)
6	11 day postoperative, 39 days old	Onset of fast	0.16 (0.13–0.18)
8	11 day postoperative, 39 days old	Fasted, 0.5 mg. G. H. at 0 and 12 hours	0.15 (0.11–0.19)
7	11 day postoperative, 39 days old	Fasted, uninjected	0.15 (0.12–0.18)

[1] Mean value and range of variation.

SUMMARY

The glycogen content of the tibia from young normal, hypophysectomized, and growth hormone-treated hypophysectomized rats has been presented. The glycogen content of the tibia from immature hypophysectomized rats was found to decrease after operation more markedly than in unoperated animals of corresponding age, and reached a constant low level about two weeks postoperative. By treating hypophysectomized rats with growth hormone daily for one to three weeks after operation, it was possible to maintain the glycogen content of the tibia at normal or higher levels. The administration of growth hormone to hypophysectomized rats already possessing a low content of tibia glycogen increased this to normal when given in sufficiently large doses. Twenty-four hours of fasting did not further alter the low glycogen content of the tibia in hypophysectomized rats, nor was there any detectable change when growth hormone was administered during the fast.

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THE ENTEROHEPATIC CIRCULATION OF THYROXINE IN THE RAT[1]

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IT WAS reported previously that, in the rat, thyroxine is excreted primarily by way of the bile and that the excretion product is a thyroxine-containing compound which we have designated "Compound U" (Taurog, Briggs, and Chaikoff, 1951, 1952). This compound is the normal, physiological excretion product of thyroxine in the rat, and has tentatively been identified as the glucuronide of thyroxine.

The existence of such a mechanism for the excretion of thyroxine raises the question as to its effect on the *reabsorption* of the hormone from the gut. In our earlier work, it was found that a large fraction of injected thyroxine appeared in the feces. This finding, together with the observation that there is no discernible Compound U in rat plasma, led us to suspect that thyroxine, in the form of Compound U, is not well absorbed from the intestinal tract.

The present report describes some experiments designed to study more directly the enterohepatic circulation of thyroxine and the manner in which it is affected by the conversion of thyroxine to Compound U.

EXPERIMENTAL

The general design of the experiments was as follows: Bile was collected by fistula from 2 groups of *donor* rats: 1) rats injected with 100 to 125 μg. of I^{131}-labeled L-thyroxine (rats A–E, Table 1); 2) untreated rats (rats F–L, Table 1). The bile obtained from the untreated rats was made to contain known amounts of either I^{131}-labeled L-thyroxine or I^{131}-labeled Compound U by the addition of purified samples of these compounds (Table 1). The bile samples prepared as described above were then dripped slowly, via the bile duct, into the duodenums of *recipient* rats. (The treatment of both donor and recipient rats is shown in Table 1.) The bile produced by the recipient rats was collected by fistula, analyzed for total I^{131}, and, where the amount of activity was sufficient, fractionated by the method of filter paper chromatography. The details of these procedures are

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TABLE 1. TREATMENT OF DONOR AND RECIPIENT RATS

Recipient rat			Donor rats from which bile was obtained	Treatment of donor rats	I^{131}-compounds in infused bile
No.	Volume of bile infused	Interval during which bile infused			
	cc.	hr.			
1	8	6	A–C (pooled bile)	Injected with 100 μg. radiothyroxine	Compound U (62%); thyroxine (36%)
2	8	6	A–C (pooled bile)	Injected with 100 μg. radiothyroxine	Compound U (62%); thyroxine (36%)
3	8	6	A–C (pooled bile)	Injected with 100 μg. radiothyroxine	Compound U (62%); thyroxine (36%)
4	10	6.5	D	Injected with 125 μg. radiothyroxine	Compound U; thyroxine*
5	10	3	E	Injected with 100 μg. radiothyroxine	Compound U; thyroxine*
6	10	7	F	None	Thyroxine (added)
7	10	7	F	None	Thyroxine (added)
8	11	6	G	None	Thyroxine (added)
9	8	4	H	None	Thyroxine (added)
10	20	9	J	None	Compound U (added)
11	13	6	K	None	Compound U (added)
12	10	7.5	L	None	Compound U (added)

* The bile which was infused into rats 4 and 5 contained much more Compound U than thyroxine, as shown by the radio-autograms of the chromatograms. Quantitative estimates of the relative amounts were not made, however.

described below. Intestinal absorption of the infused I^{131} was determined in the recipient rats by the difference between administered I^{131} and I^{131} appearing in feces and gastrointestinal contents.

Preparation of I^{131}-Labeled L-Thyroxine.—L-thyroxine (Glaxo Laboratories)[3] was incubated at 60° with I^{131}-labeled molecular iodine (I_2^{131}) in 50 per cent ethanol solution at pH 4–5. After 30–60 minutes the thyroxine was precipitated by the addition of water, centrifuged, and washed several times with water. The thyroxine crystals were dissolved in a small amount of 0.02N NaOH, and the desired volume made with isotonic $NaHCO_3$. The specific activity of the final product was generally about 2 μc. per μg. of a purity previously described (Taurog, Briggs, and Chaikoff, 1952).

I^{131}-activity in the various tissues of the rat was determined by dissolving the tissue in strong alkali, drying a small aliquot in a procelain dish, and counting the β-radiation with an end-window Geiger tube. Because of the limitations of this procedure, measurements of I^{131} in the rat carcasses were subject to considerable error, and in the cases where the amount of I^{131} administered to the recipient rats was relatively small, no measurements of carcass-I^{131} are reported.

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[3] We are indebted to Dr. B. A. Hems, Research Division, Glaxo Laboratories, Ltd., for a generous supply of L-thyroxine.

RESULTS

I. *Experiments with Bile Obtained from Donor Rats Injected with I^{131}-Labeled Thyroxine*

Three donor rats (A–C, Table 1) were injected subcutaneously with approximately 100 µg. of I^{131}-labeled L-thyroxine, and their bile ducts were cannulated for bile collection as described elsewhere (Taurog, Briggs, and Chaikoff, 1951). The bile samples obtained during the interval 3–12 hours after the injection were pooled and saved for injection into recipient rats. Analysis of this bile by the method of filter paper chromatography (Taurog, Tong, and Chaikoff, 1950) showed that Compound U accounted for 62 per cent, and thyroxine for 36% of the total radioactivity (Fig. 1a).

To study the enterohepatic circulation of these compounds, 3 recipient rats were treated as follows: Two cannulae were inserted into the bile duct: one was placed in the proximal, or hepatic, end of the duct, and the other,

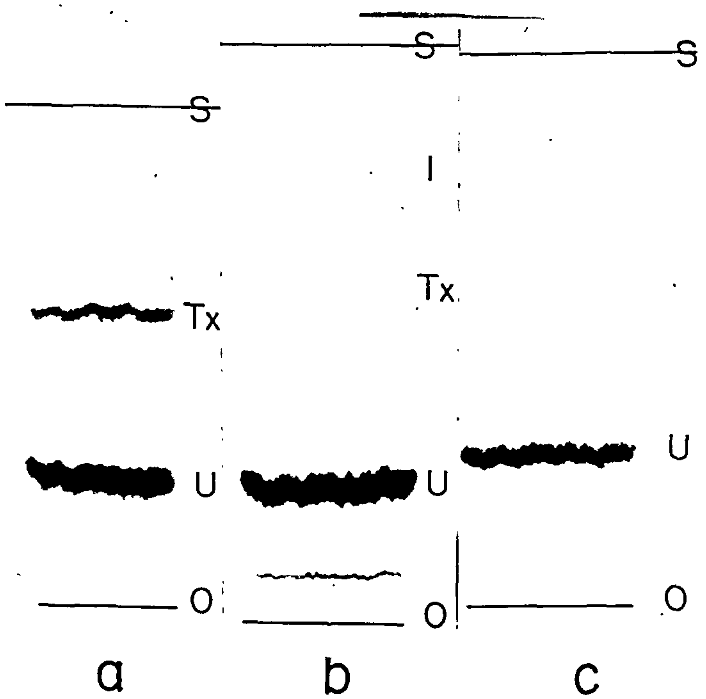

Fig. 1. Radioautograms of ascending filter paper chromatograms: a) Bile from donor rats, A–C, used for infusion into recipient rats, 1–3; b) Bile collected from recipient rat 7, 7.5–18.5 hours after infusion of bile containing added I^{131}-thyroxine; c) Bile containing added Compound U for infusion into recipient rat 10, prepared as described in text. Solvent, collidine-water (100:35) in an ammonia atmosphere. O, origin; U, Compound U; Tx, thyroxine; I, inorganic iodide; S, solvent front.

in the distal, or duodenal, end (Siperstein, Hernandez, and Chaikoff, in press). The cannula in the distal segment was attached to the barrel of a 10-cc. syringe through a drip apparatus. 8 cc. of the bile obtained from the donor rats referred to above were added to each of the 10-cc syringes. The height at which the syringe was held served to control the rate at which the bile dripped into the rat, and this was made to approximate the rate at which the recipient rat produced bile. Each recipient rat received bile for a period of 6 hours, whereas the bile was collected from this same rat for 65 hours. During that 65 hours, bile was collected serially in order to determine the rate as well as the extent of the recirculation of I^{131}.

The rate of appearance of the I^{131} in bile of recipient rats 1–3 is shown in Figure 2A. Over the entire period of 65 hours, only 5–15% of the administered I^{131} appeared in bile. The greatest part of this was secreted during the first 15 hours. In 2 other recipient rats (4 and 5, Fig. 2A) infused with bile from separate donor rats (D and E, Table 1), 9.3 and 7.6% of the administered I^{131} appeared in the bile in 41 and 51 hours, respectively. Thus it would appear that after an injection of I^{131}-labeled thyroxine only about 10% of the I^{131} excreted into the bile undergoes enterohepatic circulation.

The fate of the rest of the I^{131} that recipient rats 1–3 had received is shown in Table 2. By far the largest fraction of the I^{131} infused into the duodenums of the recipient rats was found in the feces (64–76%). It is unlikely, on the basis of previous work by Gross and Leblond (1947), that any appreciable part of the fecal I^{131} represents iodine which had been absorbed and subsequently resecreted through the intestinal wall. The large fraction of the I^{131} appearing in the feces, therefore, makes it evident that approximately 70% of the infused I^{131} was not absorbed. Of the 30% that was absorbed, only about one third was recirculated into bile.

TABLE 2. FATE OF ENTERALLY ADMINISTERED I^{131} IN RECIPIENT RATS
(The rats recorded below are the same as those referred to in Figure 2. All values refer to percentages of the administered I^{131})

Recovery	Infused with bile obtained from I^{131}-thyroxine-injected donor rats			Infused with bile containing added I^{131}-thyroxine				Infused with bile containing added I^{131}-Compound U		
	Rat 1 65 hrs.	Rat 2 65 hrs.	Rat 3 65 hrs.	Rat 6 64 hrs.	Rat 7 64 hrs.	Rat 8 37½ hrs.	Rat 9 67 hrs.	Rat 10 68½ hrs.	Rat 11 67½ hrs.	Rat 12 49 hrs.
	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}
Bile	10.9	14.7	5.5	20.6	19.5	17.1	31.0	20.3	9.6	12.2
Feces + gastrointestinal contents	64.6	64.0	75.8	58.8	43.4	44.0	31.8	67.5	75.8	60.0
Urine*	2.0	0.8	2.2	3.0	2.5	4.6	3.6	0.3	0.9	0.7
Gut	0.5	0.7	0.1	0.3	0.5	0.7	2.9	†	1.8	2.1
Thyroid	†	†	†	3.1	3.7	3.8	6.8	4.7	2.6	2.7
Carcass	11.7	18.3	4.6	11.5	15.4	‡	‡	‡	‡	‡

* The urine values are only approximate since the urine samples were contaminated with feces.
† Measurement not made.
‡ Concentration of I^{131} too low for measurement.

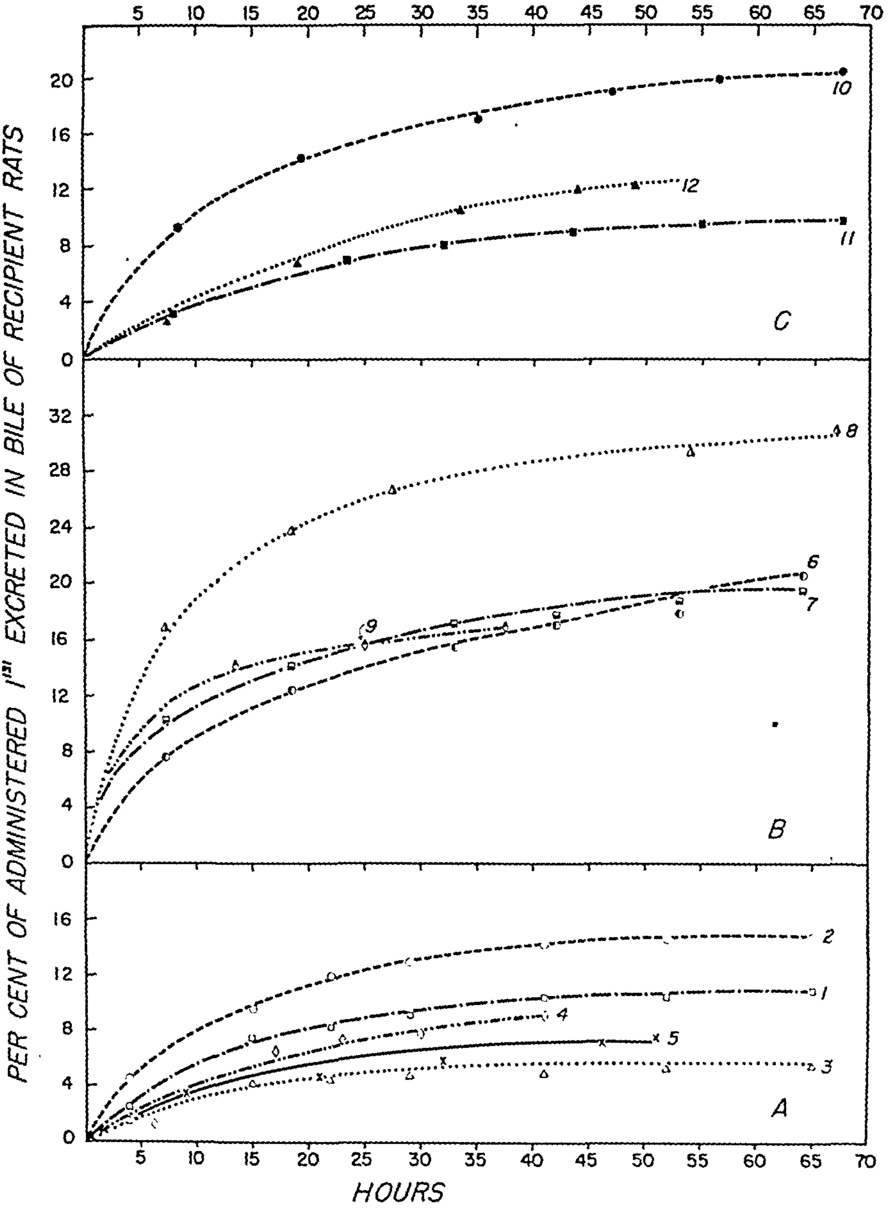

Fig. 2. Cumulative excretion of I^{131} in bile of recipient rats: A) Rats 1–5, infused with bile obtained from I^{131}-thyroxine injected rats; B) rats 6–9, infused with bile containing added I^{131}-thyroxine; C) rats 10–12, infused with bile containing added I^{131}-Compound U.

II. Experiments with Bile to Which I^{131}-Labeled L-Thyroxine Was Added

The experiments described above were performed with bile containing both thyroxine and Compound U. It became of interest to determine whether the enterohepatic circulation of these 2 compounds differed, and the experiments designed to test this point are described in the present and following sections.

Nonradioactive bile was collected from donor rats that had *not* been injected with I^{131} (F–H, Table 1). To this bile was added I^{131}-labeled L-thyroxine, prepared as described above. 8–11 cc. of such thyroxine-containing bile were then infused slowly into the duodenums of 4 recipient rats via the bile duct, just as described in Section I. Rats 6 and 7 received, in this manner, approximately 8 μg. of thyroxine iodine, whereas rats 8 and 9 were infused with approximately 0.9 and 0.5 μg. of thyroxine iodine, respectively. Bile was collected from rats 6 and 7 for 64 hours, and from rat 8 for 67 hours. Rat 9 removed its bile cannula after 37.5 hours, and the bile collection was therefore terminated at that interval.

The rate of appearance of the I^{131} in the bile of the recipient rats is shown in Figure 2B. 17–31% of the infused I^{131} appeared in bile during the collection periods. These values are significantly higher than the corresponding values observed for rats 1 to 5 ($p < 0.01$). The biliary excretion of the administered I^{131} by rats 6 and 7, which received 8 μg. of thyroxine iodine, did not differ appreciably from that by rats 8 and 9 which received one-tenth as much thyroxine.

The bile collected from rats 6 and 7 was analyzed by the method of filter paper chromatography. The radioautograms (Fig. 1b) show that the thyroxine which recirculated into the bile was present primarily in the form of Compound U. Only a very small part of the I^{131} appearing in bile was unchanged thyroxine. The bile from rats 8 and 9 did not contain a sufficient concentration of I^{131} to permit analysis by paper chromatography.

The fate of the remainder of the thyroxine I^{131} infused into recipient rats 6 to 9 is shown in Table 2. The largest fraction of the I^{131} was again found in the feces. However, the total absorption of I^{131} was 41–68%, significantly higher ($p \sim .02$) than the absorption of I^{131} in rats 1–3 (24–36%, Table 2).

III. Experiments with Bile to Which I^{131}-Labeled Compound U Was Added

Purified samples of Compound U were obtained in the following manner: A rat was injected subcutaneously with 100–150 μg. of I^{131}-labeled L-thyroxine, and its bile duct was cannulated. The bile was collected during an interval when the concentration of Compound U was maximum (e.g., 8 to 14 hours). It was brought to pH 3.5–4.0 with dilute HCl, and extracted with an equal volume of n-butanol. A total of 200 μl. of the butanol extract was delivered in 25 μl. portions on each of 24 filter paper strips. Compound U was separated from other I^{131}-containing components of bile by chromatography in collidine-water-NH_3, as previously described (Taurog, Briggs, and Chaikoff, 1952). The sections of the filter paper chromatograms corresponding to Compound U were cut out, and each was eluted with approximately 0.3 ml. of .02N NaOH. The eluates of the 24 strips were combined and concentrated to a volume of approximately 1 ml. *in vacuo* at about 45°.

Three samples of Compound U were prepared as described above. The quantity of Compound U iodine was estimated from its radioactivity and from the known specific activity of the injected thyroxine iodine, on the assumption that the specific activity of the Compound U iodine was very close to that of the injected thyroxine iodine. The Compound U samples were added to nonradioactive bile obtained from donor rats J–L, for infusion into recipient rats 10–12. Rat 10 received approximately 0.5 μg. of Compound U iodine in 20 cc. of bile; rat 11 received approximately 1.5 μg. of Compound U iodine in 10 cc. bile; and rat 12, approximately 2 μg. of Compound U iodine in 13 cc. bile. The infused bile samples were analyzed by paper chromatography. At least 90% of the I^{131} was present in the form of Compound U (Fig. 1c).

The rate at which the infused Compound U-I^{131} appeared in the bile of the recipient rats is shown in Figure 2C (rats 10–12). Values of 9.6, 12.2, and 20.3% of the administered I^{131} were obtained in 49 to 68 hours. These values appear to be somewhat lower than those obtained after the infusion of bile containing I^{131}-thyroxine (rats 6 to 9, Fig. 2B), although the difference between the mean values is not statistically significant ($0.2 > p > 0.1$).

The fate of the remainder of the I^{131} in rats 10–12 is shown in Table 2. By far the largest fraction of the administered I^{131} appeared in the feces 60–76%). The absorption of I^{131} in these rats (24–40%) was significantly less ($p \sim 0.02$) than the absorption of I^{131} in the rats which received labeled thyroxine (41–68%). It may be concluded, therefore, that Compound U is less readily absorbed than thyroxine from the gastrointestinal tract of the rat.

DISCUSSION

It has been demonstrated by many investigators, using rabbits (Elmer and Luczynski, 1933), cats (Clayton *et al.*, 1950), and dogs (Asimow and Estrin, 1931; Schittenhelm and Eisler, 1932), that a significant portion of an enterally administered dose of thyroxine iodine appears in the bile. The nature of this iodine could not be determined with certainty by previous methods, but several workers have suggested the possibility of an enterohepatic circulation of thyroxine (Leblond, 1951; Albert *et al.*, 1952).

The method of filter paper chromatography has now made it possible to separate thyroxine from other closely related iodine compounds. The application of this method of analysis to bile collected from rats injected with small doses of I^{131}-labeled thyroxine has revealed that, although some unchanged thyroxine is present, most of the I^{131} is in the form of a conjugated thyroxine compound which we have called Compound U.

In the experiments reported here, we have attempted to measure the intestinal absorption and the enterohepatic circulation of thyroxine and of its excretion product, Compound U. Bile obtained from rats injected with 100 to 125 μg. I^{131}-thyroxine, and containing I^{131} in the form of both Compound U (approximately $\frac{2}{3}$) and thyroxine (approximately $\frac{1}{3}$), was slowly infused, via the bile duct, into the duodenums of recipient rats. Approximately 30% of the I^{131} was absorbed. The bile secreted by the recipient rats contained 5.5 to 14.7 (average 9.6%) of the administered I^{131}, or approximately $\frac{1}{3}$ of the absorbed I^{131}. From these data it may be concluded that of the iodine which is secreted into the bile of a rat following the injection of thyroxine, only about 10% undergoes enterohepatic circulation.

Experiments were carried out to determine whether there was any appreciable difference between unchanged thyroxine and Compound U in the extent of their absorption and enterohepatic circulation. In these experiments, purified samples of I^{131}-thyroxine or of I^{131}-Compound U were added to nonradioactive bile. The bile prepared in this manner was then infused into the duodenums of recipient rats from which bile was also collected. The rats that received bile containing added I^{131}-thyroxine absorbed 41–68 (average 58) % of the administered I^{131}, significantly more than the rats that received bile containing I^{131}-Compound U. In both groups, $\frac{1}{3}$ to $\frac{1}{2}$ of the absorbed I^{131} was recirculated into the bile, mostly in the form of Compound U.

It does not seem likely that absorption of Compound U depends on its hydrolysis to thyroxine. It was reported previously (Taurog, Briggs, and Chaikoff, 1952) that Compound U is not hydrolyzed by the enzymes of the upper intestinal tract, although it is hydrolyzed by the contents of the lower bowel.

The finding that Compound U is less readily absorbed from the gastrointestinal tract than is thyroxine provides added evidence that the conversion of thyroxine to Compound U is a mechanism for ridding the organism of excess thyroxine more efficiently. It is pointed out elsewhere (Briggs, Brauer, Taurog, and Chaikoff, in press) that the liver secretes Compound U into the bile more readily than it does unchanged thyroxine, and that the conversion of thyroxine to Compound U in the rat is, therefore, probably a simple detoxification reaction. This detoxification mechanism, however, is quite limited in the rat, and seems to break down when very large doses of thyroxine are administered.

In a recent paper, Albert *et al.* (1952) reported on the absorption of thyroxine from isolated portions of the gastrointestinal tract of the rat. Using I^{131}-labeled L-thyroxine obtained from Abbott Laboratories, these workers reported 20 to 25% absorption from the small bowel. These values are somewhat lower than the ones observed here.

SUMMARY

1. The absorption and enterohepatic circulation of thyroxine and its excretion product, Compound U, were studied in the rat.

2. Bile obtained from rats injected with 100 to 125 μg. of I^{131}-labeled L-thyroxine was infused slowly, via the bile duct, into the duodenums of recipient rats. The I^{131} in the infused bile, which was largely in the form of Compound U, was absorbed to the extent of 25 to 35%. 5.5 to 14.7% of the administered I^{131} appeared in the bile secreted by the recipient animals in 65 hours.

3. I^{131}-labeled L-thyroxine was added to nonradioactive bile and infused slowly, via the bile duct, into the duodenums of recipient rats. The absorption of the thyroxine iodine varied from 41 to 68%. The I^{131} which appeared in the bile secreted by the recipient rats varied from 17 to 31% of the administered I^{131}.

4. I^{131}-labeled Compound U, isolated by elution from filter paper chromatograms, was added to nonradioactive bile and infused slowly into the duodenums of recipient rats. The absorption of the iodine varied from 24 to 40%. The I^{131} which appeared in the bile of the recipient rats varied from 9.6 to 20.3%.

5. Compound U is less readily absorbed from the gastrointestinal tract than is thyroxine. The conversion of thyroxine to Compound U in the rat therefore permits the animal to eliminate excess thyroxine somewhat more efficiently.

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THE EFFECTS OF IODIDE, PERCHLORATE, THIOCYANATE, AND NITRATE ADMINISTRATION UPON THE IODIDE CONCENTRATING MECHANISM OF THE RAT THYROID*

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THE thyroid gland of an animal chronically treated with a thiouracil-like drug, although unable to manufacture thyroid hormone, manifests a remarkable ability to collect and concentrate the iodide ion (Vanderlaan and Vanderlaan, 1947; Taurog, Chaikoff, and Feller, 1947). About one hour after the subcutaneous administration of iodide in rats the thyroid exhibits an average concentration gradient of 250 over the serum. Taurog, Chaikoff, and Feller (1947) noted this ratio to be unchanged when 2 μg. or 100 μg. of iodide were given to adult rats. Vanderlaan and Vanderlaan (1947) found that the gradient remained constant when doses up to 100 μg. of potassium iodide were administered in young rats, but that when larger quantities were given it diminished progressively. However, even when 10,000 μg. were given the gradient was not abolished completely, a concentration of 3 fold persisting in favor of the gland.

In contrast to the action of the thiouracil-like agents, the antithyroid action of thiocyanate is characterized by interference with thyroidal collection of inorganic iodide (Franklin, Chaikoff, and Lerner, 1944). Thiocyanate has also been shown to discharge iodide previously accumulated by the thyroid gland of the rat treated with propylthiouracil (Vanderlaan and Vanderlaan, 1947). Recently, it has been found (Wyngaarden, Wright, and Ways, 1952) that certain monovalent anions, principally the halogen oxides, share with thiocyanate the properties of inhibiting collection and of interfering with retention of the iodide ion within the thyroid gland. Perchlorate, the most potent anion of this group, was 10 times as effective as thiocyanate in this capacity. The effectiveness of perchlorate as an

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iodide discharging agent and as an uptake inhibitor has also been demonstrated in the thyroid patient (Stanbury and Wyngaarden, 1952).

The present report describes studies upon the comparative effects of graded "carrier" doses of four agents, perchlorate, thiocyanate, inert iodide, and nitrate, upon the ability of the thyroid gland to maintain a concentration gradient of radioiodide over the serum.

METHODS

Male Wistar rats in groups of 24–36 animals, having group average weights of 155–196 gm., were treated for 14 or more days with the antithyroid regimens specified in Table 1. In most experiments, one hour prior to the administration of radioiodide the rats received 10 mg. of propylthiouracil intraperitoneally to supplement the dietary antithyroid agent. Ten μc. of radioiodide were then administered subcutaneously in 1 cc. of water, which also contained the desired quantity of the "carrier" anion. Potassium iodide as carrier was given in doses up to 10,000 μg. per rat. The other compounds were administered in molar equivalents of specific dose levels of potassium iodide. One hour following the administration of radioiodide the rats were killed with sodium pentobarbital administered intraperitoneally. Blood was withdrawn from the inferior vena cava and the serum separated. Thyroid glands were removed, weighed on a Roller-Smith balance, and digested in 3 cc. of 5% potassium hydroxide containing 10 μg. of sodium iodide. Two-tenths cc. of serum or of thyroid digest were then evenly distributed upon silvered copper planchets, and their radioactivity was determined with the Geiger-Muller counter. The ratio of radioactivity between thyroid and serum was determined for each rat, from the activity data per gram of thyroid and per cc. of serum (without correcting for specific gravity). No correction was applied for the variations of average weight from group to group.

RESULTS

In Table 1 are given the individual thyroid-serum ratios of radioiodine and the gradient averages at each dose level for each group. The minimal dose levels effecting statistically significant reductions of the average collection gradients, expressed in micrograms of potassium iodide, or in equivalent doses of other agents, are: KI, 1000; KSCN, 1000; KNO_3, 1000; $KClO_4$, 30.

The average gradients have also been expressed as percentages of the average control gradient. From these values composite averages, adjusted in each computation for the size of the series, have been calculated for each compound, and these results are plotted in Figure 1. It can be seen that perchlorate was distinctly more potent than the other ions. Not only was a significant reduction of the gradient produced by a relatively small dose of this substance, but in the rats receiving potassium perchlorate in doses equivalent to 10,000 μg. of potassium iodide, the radioiodide accumulation gradient was virtually abolished.

The approximately equal effectiveness of molar equivalent doses of iodide and of thiocyanate is to be noted. This result had been anticipated

TABLE 1. RATIO OF THYROID RADIOACTIVE IODIDE TO SERUM RADIOACTIVE IODIDE IN RATS INJECTED WITH INCREASING DOSES OF POTASSIUM IODIDE AS CARRIER, OR OF EQUIVALENT DOSES OF THE OTHER AGENTS, ACCOMPANYING A CONSTANT TRACER DOSE OF RADIOACTIVE IODIDE

Compound	Group	Pretreatment*	Micrograms of potassium iodide, or equivalent dose of other agent											
			0	3	10	30	100	300	1,000	3,000	10,000	30,000	100,000	300,000
KI	I	Reg. TU	93				136	112	51		8			
			66				59	78	66		8			
			63				50	42	55		11			
			50				139	102	91		6			
			45				79	68	46		9			
							115	71	40		11			
			Av. 63 ±5.6‡ (100%)				96 ±10.7 (152%)	79 ±6.8 (125%)	58 ±5.0 (92%)		9±.5 (14%)			
	II	Reg. TU	61			73		50	43	13	9			
			96			121		64	32	24	10			
			87			92		39	67	17	11			
			42			38		78	26	25	8			
			36			90		75	41	15				
			Av. 64±8.0			83±9.2 (130%)		61 ±5.0 (95%)	42 ±4.7 (66%)	19 ±1.6 (30%)	10±.5 (16%)			
	III	Reg. TU†	124		65		93	80	79	19	9			
			98		166		138	77	89	28	12			
			83		118		112	84	54	20	12			
			136		12		78	113	97	22	16			
								127	100	49				
			Av. 110±8.1		90±22.4 (82%)		108 ±7.8 (98%)	96 ±6.8 (87%)	84 ±5.6 (76%)	28 ±4.9 (25%)	12±1.4 (11%)			
	Total Av.		100%		82%	130%	129%	104%	79%	28%	14%			
KSCN	I	Reg. TU†	250			377		412	327	26	37			
			242			447		301	108	42	102			
			419			135			83	141	19			
						348			130					
			Av. 330±40.9			327±45.3 (99%)		356 (108%)	162± ±37.6 (49%)	70 ±24.1 (21%)	56±17.1 (17%)			
	II	Rem. TU†	206			79		168	42	29	14			
			131			131		76	36	11	6			
			61			40		40	54	23	22			
			43			51		35	77	23	5			
			85			89		83	57	12	3			
			113			135		40	32	19				
			Av. 107±16.1			88±10.9 (82%)		74 ±13.9 (69%)	50 ±4.6 (47%)	20 ±1.9 (19%)	10±2.4 (9%)			
	III	Rem. TU†	152			204		131	76	42	4			
			201			254		77	82	36	6			
			213			71		30	180	67	5			
			91			359		47	54	41	4			
			216			96		254	86	10	12			
									222	94				
			Av. 175±16.1			197±35.5 (113%)		108 ±27.3 (62%)	115 ±18.6 (66%)	48 ±7.9 (27%)	6±1.0 (3%)			
	Total Av. %		(100%)			(97%)		(72%)	(55%)	(23%)	(6%)			
KClO₄	I	Rem. PTU	214			145	56	15	5	3	2			
			148			102	115	27	8	3	2			
			265			59	72	17	7	5	2			
			181			106	41	30	7	5	1			
			119			69	31	19	8	4				
			Av. 185±17.2			96 ±10.3 (52%)	63 ±9.8 (34%)	22 ±2.0 (12%)	7 ±0.4 (4%)	4 ±0.3 (2%)	2±0.2 (1%)			

TABLE 1—*Continued*

Compound	Group	Pretreatment*	Micrograms of potassium iodide, or equivalent dose of other agent											
			0	3	10	30	100	300	1,000	3,000	10,000	30,000	100,000	300,000
$KClO_4$	II	Rem. PTU	301	291	209	104	82		8					
			124	176	209	185	82		12					
			358	138	176	155	57		13					
			271	136	300	168	27		13					
				429	144	61	67		11					
			Av. 264 ±33.4	215 ±36.8 (82%)	208 ±17.6 (79%)	135 ±15.4 (51%)	63±9.9 (24%)		11±0.6 (4%)					
Total Av. %			(100%)	(82%)	(79%)	(52%)	(29%)	(12%)	(4%)	(2%)	(1%)			
KNO_3	I	Rem. PTU	207			313		203	192	180	245			
			168			297		74	142	136	116			
			256			62		194	29	86	42			
			238			89		72	28	63	193			
			81			142		60	112	56	39			
			Av. 190±21.0			181±35.4 (95%)		122 ±21.0 (64%)	101 ±21.6 (53%)	104 ±15.9 (55%)	127 ±27.5 (67%)			
	II	Rem. PTU	521				218		394		291	110	36	25
			251				456		159		157	248	42	18
			209				222		161		261	135	31	17
			398				176		112		132	120	17	31
							131		105		191	47	39	7
			Av. 345±48.0				241±30.9 (70%)		186±36.0 (54%)		206 ±20.8 (60%)	132 ±22.0 (38%)	33 ±3.0 (10%)	20 ±2.7 (6%)
Total Av. %			(100%)			(95%)	(70%)	(64%)	(54%)	(55%)	(64%)	(38%)	(10%)	(6%)

* [illegible]

/100 gm.).

†

‡ Averages represent: Mean± probable error of the mean (R). $R = .6745 \sqrt{\frac{\Sigma(V^2)}{n(n-1)}}$.

(Stanbury, 1951) from a comparison of the data of Vanderlaan and Vanderlaan (1947) concerning the effects of carrier iodide upon the concentrating mechanism, with those of Raben and Astwood (1949) concerning the effects of thiocyanate.

It is apparent that considerably larger equivalent doses of potassium nitrate are required to reduce the gradient to 10% or less of the control level than of the other compounds, despite the fact that in terms of minimal effective dose, nitrate is similar to iodide and thiocyanate. It is noteworthy (Table 1) that some rats appear to be quite resistant to potassium nitrate even in doses of 6–18 mg., the equivalents of 10,000 to 30,000 μg. of potassium iodide.

DISCUSSION

These results are quantitatively in general agreement with those concerning the effectiveness of perchlorate, thiocyanate and nitrates as dischargers of thyroidal iodide in the intact rat (Wyngaarden, Wright, and Ways, 1952). In the present study, the order of effectiveness of these agents

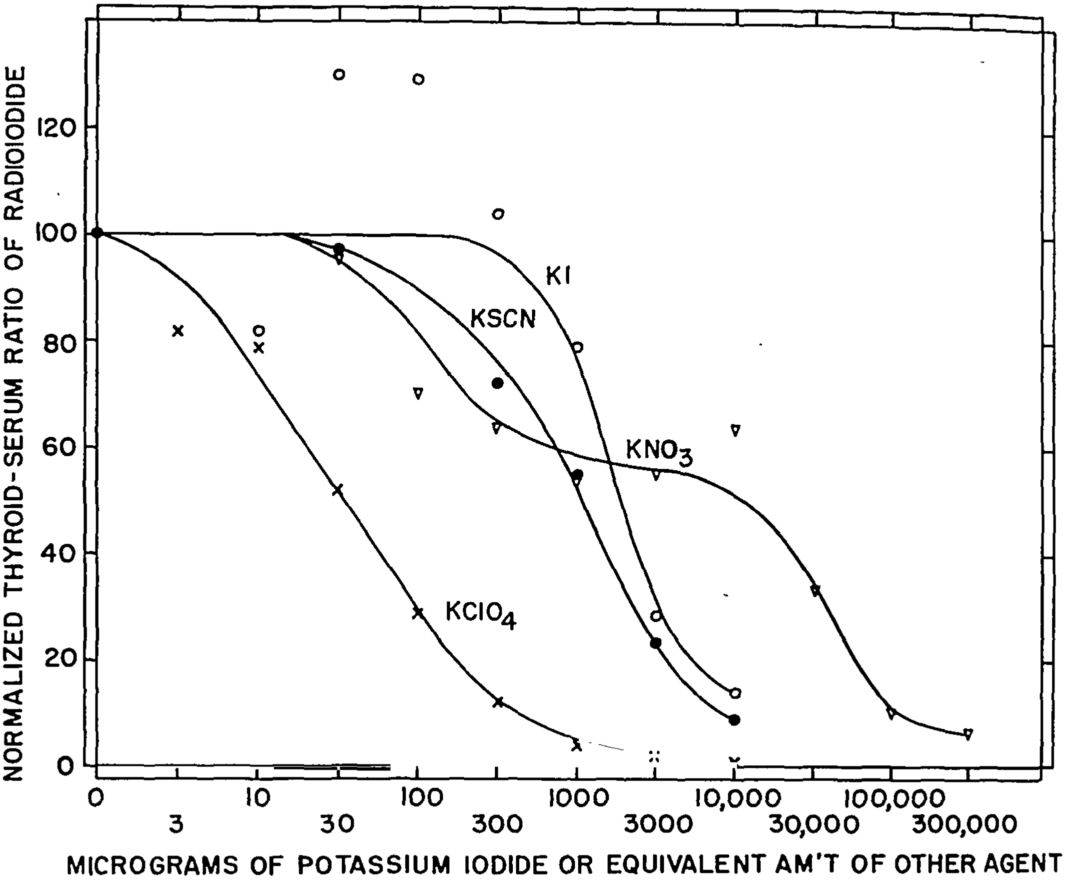

FIG. 1. The effects of iodide, thiocyanate, perchlorate, and nitrate ions upon the concentration gradient of I^{131}, when these agents were given in increasing doses, accompanying a constant dose of I^{131}. The ordinate is thyroid-serum I^{131} ratio, normalized to express the control gradient for each group as 100, and averaged for each agent. The abscissa is the dose of potassium iodide or equivalent dose of thiocyanate, perchlorate, or nitrate, plotted logarithmically.

in producing a profound depression of the accumulation gradient is: perchlorate, thiocyanate, iodide and nitrate. In contrast to perchlorate, thiocyanate and nitrate, which reduce simultaneously the concentration gradient and the total iodide content of the gland due to an inhibition of uptake, iodide exerts its effect upon the gradient by leading to a partial saturation of the collecting mechanism (Vanderlaan and Vanderlaan, 1947).

Since the efficiency of any anion in discharging iodide *in vivo* will necessarily depend in part upon its extra-thyroidal disposal rate, studies of the effects of these anions upon tissue slices under conditions of controlled concentration will be necessary to establish more exactly the relative potencies of antithyroid action. The present evaluations, obtained in the intact animal, may be subject to revision when such data are available.

The mechanism of action of these agents has not been established. Simple competition for collection by the thyroid cannot account for the action of this anion group, since thyroidal accumulation of thiocyanate ion has been excluded by Wood and Kingsland (1950), and that of another antithyroid anion, iodate, by Leblond and Sue (1941). The preferential inhibition of thyroid function caused by these ions is apparently not accompanied by preferential concentration. Yet the effect of excess iodide in abolishing the antithyroid action of thiocyanate (Astwood, 1943) and of overcoming the interference with iodide collection caused by perchlorate (Wyngaarden, Wright, and Ways, 1952) suggests that a competitive mechanism is involved.

In preliminary studies on the effects of graded carrier doses of potassium iodide, rats were employed which had been made goitrous either by using a fox chow or a Remington low iodine diet to which had been added either thiourea or thiouracil (cf. Table 1). These regimes resulted in comparable degrees of thyroidal enlargement. The mechanism of action of thiouracil and propylthiouracil are considered to be identical. The quantities of iodide consumed daily by the animals on the Remington or the fox chow diet were far below the smallest dose of carrier iodide which resulted in any change in the thyroid-serum iodide ratio. It would appear that the effects of the anions studied were independent of the regimen employed for goiter production.

CONCLUSIONS

The effects of increasing quantities of inert iodide, thiocyanate, perchlorate and nitrate upon the ability of the thyroid gland to concentrate radioiodide have been studied in the rat. All of these agents caused a marked depression of the iodide concentration gradient existing between thyroid and serum, when given in sufficient quantities. The most potent antithyroid anion was perchlorate, the least, nitrate. The effects of molar equivalent doses of inert iodide and of thiocyanate upon this gradient were approximately equal. The mechanism of action of these anions is unknown. It is probable that the apparent qualitative similarity of perchlorate, thiocyanate, and nitrate with that of excess iodide is only superficial.

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THE ADRENAL CORTEX AND SULFUR METABOLISM: THE INFLUENCE OF AMINO ACID SULFUR ON THE FORMATION OF LIVER GLYCOGEN[1]

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A NUMBER of years ago it was demonstrated that oral or percutaneous administration of sulfur to rats or rabbits resulted in adrenal hyperplasia and marked deposition of liver glycogen (Burgi and Gordonoff, 1926; Foldes, 1928). The possible interrelation of these two observations remained obscure at that time, for the glycogenic activity of 11-oxy corticoids was as yet unsuspected. In retrospect, it seems likely that the administered sulfur induced an adrenal hyperplasia and an outpouring of adrenal cortical hormones which resulted in excessive liver glycogen deposition.

Recent studies indicating an intimate association between adrenal cortical function and sulfur metabolism (Goldzieher and Edlin, 1953; Goldzieher, Rawls and Goldzieher, 1953) have suggested further exploration of these findings. In view of the fact that colloidal sulfur, though of a very low order of toxicity, is still a foreign substance and its administration may constitute a nonspecific stress, it was decided first to investigate the effect of sulfur in physiological form. Studies were accordingly carried out with *l*-cysteine, *l*-cystine, glutathione, and the sulfurless analogue of cysteine, serine, in order to determine whether or not the presence of sulfur exercised a specific effect on the ability of these substances to cause deposition of liver glycogen and furthermore, whether or not such effects were influenced by the presence or absence of cortical hormones.

MATERIALS AND METHODS

Adult CFW male rats weighing 200 to 250 gm. were maintained on a stock diet of Purina fox checkers and water *ad lib.* The intact animals were fasted for 12, 24 or 48 hours before administration of the test substance. In other animals, adrenalectomy was performed under nembutal anesthesia; they were then maintained on 10% glucose in saline for one day, on saline and an *ad lib.* diet for one day, and fasted for 24 hours before use. The test substance was administered intraperitoneally or by stomach tube as indicated; controls received isotonic saline intraperitoneally or tap water by stomach

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THE ADRENAL CORTEX AND SULFUR METABOLISM: THE EFFECT OF ACTH ON THE SULFHYDRYL CONCENTRATION OF RAT ADRENAL, LIVER AND MUSCLE[1]

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AS EARLY as 1909 it was noted that the adrenal cortex was unexpectedly active in sulfur metabolism: both its sulfur content and its ability to store such compounds were greater than those of other tissues studied (Aufrecht and Diesing, 1909; Loeper *et al.,* 1926). In 1928 it was shown that treatment of rats and rabbits with colloidal sulfur resulted in adrenal cortical hypertrophy and sulfur storage, together with massive accumulation of liver glycogen (Lurie, 1928; Foldes, 1928). At that time, however, the changes in these two organs could not be interpreted, for the relation between liver glycogen deposition and 11-oxycorticosteroids was as yet unsuspected.

In recent years, further evidence has accumulated to show a relation between sulfur metabolism and adrenal cortical function. Studies with S^{35} for example, have confirmed the great avidity of the cortex for cystine sulfur (Lee *et al.*, 1951). Recent studies in our laboratory (Goldzieher *et al.*, in press) have demonstrated a rapid accumulation of SH in the adrenal cortex following the administration of cysteine. Conversely, ACTH and cortisone have been shown to influence blood levels of sulfhydryl (Hess *et al.*, 1951; Goldzieher *et al.*, in press) in animals and man. The incorporation of S^{35} into regenerating connective tissue is inhibited by cortisone in direct proportion to its concentration in the tissue (Layton, 1951). The urinary excretion of SH and SS sulfur is increased by the administration of ACTH (Kinsell *et al.*, 1950).

The relation of the adrenal cortex to sulfur metabolism has been under investigation by us for some time; the present report deals only with the effect of ACTH on the sulfhydryl content of the adrenals, and in comparison, with that of other tissues such as liver and muscle.

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[2] Present address: Southwest Foundation for Research and Education, San Antonio, Texas.

MATERIALS AND METHODS

Adult male CFW rats, weighing 200 to 250 Gm. were used throughout. They were maintained on a diet of Purina Fox Checkers and water *ad lib.* Food was withheld during the night prior to treatment.

ACTH[3] was prepared just before use in the single-dose experiments and daily for chronic studies. The quantities designated are in terms of International Standard. Injections of ACTH were given intramuscularly, and the animals were killed by a blow on the head three hours after the last injection. Both adrenals were dissected rapidly, freed of adherent tissue, enclosed in parafilm, and weighed to the nearest 0.1 mg. Approximately 100 mg. portions of liver and diaphragm were prepared in the same way. The tissues were ground to an emulsion in an all-glass Ten Broeck tissue grinder in 2.5 or 5.0 ml. 2.5% sulfosalicylic acid containing 0.5% disodium ethylene diamine tetraacetate. The emulsions were centrifuged for 20 minutes and the supernatant used for titration. The sulhydryl determinations were carried out by the amperometric method described by Benesch and Benesch (1950) and by Weissman, Schoenbach and Armistead (1950), as modified by us (Goldzieher and Epstein, in press) to increase the specificity and reproducibility of the method.

The dosage and duration of ACTH treatment is given in the following tables. The standard deviation and statistical significance of the data were evaluated by means of the t-test, using the formulas corrected for small groups.

RESULTS

Adrenal nonprotein sulfhydryl. The changes in adrenal sulfhydryl with varying dosage schedules of ACTH are shown in Table 1. The control value of 14.9 ± 1.6 mg.% is the result of determinations on 42 animals taken in small groups over a considerable period of time. The variation in normal adrenal sulfhydryl concentration, 10.8%, is relatively small.

The smallest dose of ACTH used, 0.1 mg., produced an increase in adrenal sulfhydryl concentration of 45%; this was of a high order of statistical significance ($p. = \ll 0.01$) and differed considerably from the response of the liver (Table 2) to the same dose. A similar reaction could not be obtained on repeating an 0.1 or 0.3 mg. dose at 8-hour intervals over 1 day,

TABLE 1. CHANGES IN RAT ADRENAL SULFHYDRYL

Treatment	Number of animals	SH concentration in mg./100 Gm. tissue (average ± st. dev.)	Significance (t-value)	P
Control	42	14.9 ± 1.6		
ACTH, 0.1 mg.; killed after 3 hrs.	6	21.6 ± 4.1	4.8	<0.01
ACTH, 0.1 mg. q. 8 hrs. ×3	6	15.5 ± 2.9	0.7	
ACTH, 0.3 mg. q. 8 hrs. ×3	5	15.2 ± 2.9	1.0	
ACTH, 4.0 mg.	4	12.4 ± 3.4	3.4	<0.01
ACTH, 4.0 mg. q. 12 hrs. ×7	4	13.1 ± 2.1	2.9	<0.01
ACTH, 0.3 mg. q. 12 hrs. ×25	3	11.7 ± 0.1	4.1	<0.01
ACTH as above, then no Rx for 5 days	3	11.3 ± 2.3	4.2	<0.01

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[3] Lot 212-103, supplied through the courtesy of Armour & Co.

TABLE 2. CHANGES IN RAT LIVER SULFHYDRYL

Treatment	Number of animals	SH concentration in mg./100 Gm. tissue (average ±st. dev.)	Significance	
			(t-value)	P
Control	4	31.3 ±3.5		
ACTH, 0.1 mg., killed after 3 hrs.	3	29.4 ±2.5	0.7	
ACTH, 0.3 mg. q. 8 hrs. ×3	2	24.1 ±0.7	2.4	0.05
ACTH, 0.3 mg. q. 12 hrs. ×25	3	23.3 ±2.6	3.0	0.03
ACTH as above, then no Rx for 5 days	3	24.0 ±2.9	2.6	0.05

for such treatment did not produce changes in adrenal sulfhydryl concentration. Large and relatively unphysiologic doses (4 mg.) or multiple doses produced a consistent and statistically significant depression of adrenal sulfhydryl concentration of the order of 12 to 24%. It is thus possible that the lack of change in adrenal sulfhydryl concentration during treatment with 0.3 to 0.9 mg. ACTH per day represents a balance point or cancellation between the SH-augmenting effect of a small dose and the SH-depressant effect of larger or multiple doses. The adrenal sulfhydryl depletion caused by 12 days of ACTH therapy was not restored after a 5-day recovery period, although the adrenal weights were once more within normal limits.

Liver nonprotein sulfhydryl. With liver tissue, the response to ACTH was not biphasic (Table 2). The small doses which produced a rise or no change in adrenal sulfhydryl concentration, caused no change and a fall, respectively, in the sulfhydryl concentration of liver tissue. Prolonged ACTH therapy caused a significant fall in the liver nonprotein sulfhydryl, just as in the adrenal, and again there was no significant recovery by the fifth day after the end of a 12-day course of ACTH treatment. These observations do not coincide with the findings of Ingbar, Otto and Kass (1951), who administered 9 mg. ACTH per day to rats for 3 and 10-day intervals, and found no change in liver nonprotein sulfhydryl after 3 days, and an actual increase after 10 days.

Muscle (diaphragm) nonprotein sulfhydryl. A few experiments were performed on rat muscle tissue (Table 3). Three or more injections of ACTH produced a significant depletion of nonprotein sulfhydryl; as with adrenal and liver tissue, there was no complete return to pretreatment levels within 5 days after the end of chronic ACTH treatment. The muscle, however, showed more of a tendency to return to normal levels than did the other tissues.

DISCUSSION

Several points are brought out by these observations. The effect on the adrenal of small doses of ACTH is entirely different from that of large doses or chronic treatment; it also differs from the response of the liver. It

TABLE 3. CHANGES IN RAT MUSCLE (DIAPHRAGM) SULFHYDRYL

Treatment	Number of animals	SH concentration in mg./100 Gm. tissue (average ±st. dev.)	Significance	
			(t-value)	P
Control	8	9.4±2.1		
ACTH, 0.3 mg. q. 8 hrs. ×3	3	6.6±0.9	2.2	0.05
ACTH, 0.3 mg. q. 12 hrs. ×25	3	4.0±1.1	4.1	<0.01
ACTH as above, then no Rx for 5 days	3	5.9±0.6	2.8	0.02

is interesting to note that this rise in adrenal nonprotein sulfhydryl coincides closely with the time at which a *drop* in blood sulfhydryl is observed in patients receiving continuous infusions of ACTH (Goldzieher *et al.*, in press).

With larger doses, the adrenal seems to behave in a nonspecific manner, for all the tissues studied undergo a depletion of nonprotein sulfhydryl. The fact that this depletion persists for some time after the end of ACTH therapy, and is found in tissues of highly diverse structure and function, indicates that it cannot be considered as a specific effect of ACTH in the same sense as the changes in adrenal ascorbic acid or adrenal cholesterol. Studies are now in progress to determine more precisely the nature of the sulfhydryl-bearing compound which is readily depleted but only slowly restored.

Anderson *et al.* (1951) have commented on the possibility that ACTH and cortisone exert their clinical effects by interfering with the sulfhydryl groups of enzyme systems. It must be noted that the sulfhydryl-bearing substances which we have studied are non-protein in nature, and are therefore not enzymes; however, they may represent the reservoir of freely-available sulfhydryl from which the SH-groups of the enzymes are replenished after engaging in the processes of oxidation-reduction associated with metabolic phenomena.

SUMMARY

Single small doses of ACTH produce what appears to be a specific reaction: a rise in the adrenal nonprotein sulfhydryl in 3 hours. Larger doses or prolonged treatment with ACTH produce a drop in nonprotein sulfhydryl which is not specific, for comparable changes also occur in liver and muscle tissue. The depletion is not restored in any of these tissues within 5 days following the end of prolonged ACTH administration.

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COMPARISON OF OVARIAN AND PITUITARY HORMONES FOR MAINTENANCE OF PREGNANCY IN PYRIDOXINE-DEFICIENT RATS[1]

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PREVIOUS studies have shown that the addition of a potent B6-antagonist, desoxypyridoxine, to a purified B6-deficient diet will result in the rapid production of a B6-deficiency in adult rats (Nelson and Evans, 1948, 1951). Acrodynia of the paws, a characteristic sign of the deficiency, and marked reproductive disturbances, namely a high incidence of resorptions, occurred after 2–3 weeks of the deficient diet. Pregnancy was maintained in such B6-deficient rats by administration of both estrone and progesterone but not by either hormone separately (Nelson, Lyons, and Evans, 1951), indicating some similarity between these B6-deficient rats and hypophysectomized-oophorectomized rats in which pregnancy can be maintained by the combination of the same hormones at the same dosage levels (Lyons, 1943). To determine whether pituitary or ovarian dysfunction is involved in the hormonal inadequacies of the B6-deficient rat, the three pituitary gonadotrophins which maintain pregnancy in the hypophysectomized rat have been tested in the vitamin-deficient rats. These three pituitary gonadotrophins are FSH, ICSH or LH, and MH (mammotrophin or lactogenic hormone, Lyons, Simpson and Evans, 1943).

EXPERIMENTAL METHODS

Stock rats of the Long-Evans strain were placed on the B6-deficient diet containing the antagonist at 80–85 days of age. This diet was the same as that used in all previous

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studies. It consisted of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco or Primex), 4% salts,[2] and 0.5 mg.% desoxypyridoxine. Crystalline vitamins per kilogram of diet were: 300 μg. d-biotin, 5 mg. 2-methyl-1, 4-naphthoquinone, 5 mg. thiamine HCl, 5.5 mg. pteroylglutamic acid, 10 mg. riboflavin, 10 mg. p-aminobenzoic acid, 20 mg. nicotinic acid, 50 mg. d-calcium pantothenate, 400 mg. inositol, and 1.0 gm. choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg. synthetic alpha-tocopherol, and 650 mg. corn oil (Mazola).

After 17 days of deficiency, the rats were bred with normal males and divided into equivalent groups as judged by body weight on the day of breeding. This experimental procedure has been shown to result consistently in 90–100% resorptions[3] in the deficient rats which usually average 21 days of deficiency prior to breeding (Nelson and Evans, 1951). One experiment was carried out in which animals with a longer period of deficiency, 28 days, was used; these rats were brought into estrus by means of one injection of 10 units pregnant mare serum (PMS). Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation had occurred. All rats were weighed at regular intervals. To minimize the question of placental hormone production, known to be so important in the latter part of pregnancy, all animals were autopsied 13 days after breeding; implantation sites, fetuses, and ovaries were carefully examined.

Injections were started 3 days after finding sperm in the vaginal smear. The steroid hormones used, estrone and progesterone, were injected subcutaneously in 0.2–0.3 cc. sesame oil. The anterior pituitary hormones used were dissolved in distilled water or physiological saline and neutralized. An FSH-ICSH combination capable of causing estrin production in the hypophysectomized rat and previously used successfully for maintenance of pregnancy in such animals (Lyons *et al.*, 1943; Lyons, 1943) was used together with both impure and purified MH preparations, which varied in activity from 10–30 I.U./mg.

Since it was possible to breed only 50% or less of the deficient animals in any large group of 30–60 rats, 5 separate experiments were carried out, 4 experiments with the standard period of 21 days of deficiency prior to breeding and one experiment with the longer period of deficiency, 28 days.

RESULTS AND DISCUSSION

Table 1 summarizes the results of testing the pituitary gonadotrophins in the B6-deficient rat. With 21 days of deficiency prior to breeding, the uninjected group showed 94% resorptions with early appearance of erythrocytes in the vaginal smear (day 10) due to early resorbing sites. The group receiving estrone and progesterone showed approximately 80% maintenance of pregnancy with normal time of appearance of vaginal erythrocytes on day 12. In contrast, the group injected with the pituitary gonadotrophins, FSH-ICSH and MH, exhibited partial maintenance of pregnancy, only ¼ of the group having living young, with early appearance of vaginal erythrocytes on day 10.

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[2] Salts No. 4 of Hegsted *et al.* (1941).

[3] The normality of reproductive performance in pair-fed controls supplemented with high levels of pyridoxine has previously demonstrated that food restriction is not a factor in the reproductive upsets occurring in this vitamin deficiency (Nelson and Evans, 1951).

To determine whether one particular hormone or all three gonadotrophins were involved, the combination of a single ovarian hormone together with the appropriate gonadotrophin(s) was next tried. Lyons (1951) has shown that when the FSH-ICSH preparation to stimulate estrogen production was injected together with progesterone, pregnancy was maintained in rats hypophysectomized after breeding; the combination of estrone with MH also maintained pregnancy in such animals (Lyons *et al.*, 1943).

In the B6-deficient rat the use of estrone together with MH gave essentially the results obtained by the use of the three pituitary gonadotrophins, namely partial maintenance of pregnancy, showing that MH stimulated ovarian production of progestin in only a few animals. The use of FSH-ICSH together with progesterone was not as beneficial, only 10% of the animals showing maintenance of pregnancy, an incidence not significantly different from that shown by animals uninjected or injected with progesterone alone (Nelson, Lyons and Evans, 1951). This may be interpreted as a failure of combined FSH-ICSH to stimulate the ovarian production of estrin in these B6-deficient rats and suggests ovarian dysfunction since the same dosage level of this hormone preparation will cause estrin production in normal immature rats and even lower doses will do so in hypophysectomized rats.

TABLE 1. EFFECT OF PITUITARY GONADOTROPHINS ON REPRODUCTION OF PYRIDOXINE-DEFICIENT RATS

Exptl. group[1]	Rats bred	Wt. change during gestation	Vaginal RBC[2]	Implantations	Implants with resorptions	Implants with living embryos	Implantation sites	
							Av. No.	Normal No.[3]
	No.	gms.	day	%	%	%		
		21 Days of Deficiency Prior to Breeding						
Uninjected	17	−9	10.1	94	94	6	9.1	
120–160 μg. FSH-ICSH +70–125 I.U. MH	15	+8	10.2	100	73	27	10.0	5.2
0.5 μg. Estrone +96–125 I.U. MH	9	+5	10.2	100	78	22	10.1	3.5
120 μg. FSH-ICSH +4 mg. Progesterone	10	−4	11.0	100	90	10	10.7	
0.5 μg. Estrone +4 mg. Progesterone	29	−2	12.1	97	21	79	9.0	5.2
		28 Days of Deficiency Prior to Breeding[4]						
Uninjected	9	−3	9.0	22	100	0	8.0	
200 μg. FSH-ICSH +75 I.U. MH	9	−6	11.4	78	71	29	7.1	4.0
0.5 μg. Estrone +4 mg. Progesterone	9	+1	13.0	89	0	100	5.8	3.5

[1] Injections were started 3 days after breeding. All animals were autopsied 13 days after breeding.

[2] This column shows the average time in days for the first appearance of erythrocytes (RBC) in the vaginal smear.

[3] This column gives the number of living embryos per litter, i.e. the number of normal implantation sites in rats with maintenance of pregnancy. Values are given only for groups in which at least 2 rats maintained pregnancy.

[4] These rats were brought into estrus by means of one injection of pregnant mare serum (10 units).

An additional experiment was carried out to check on the beneficial effects of the pituitary gonadotrophins. A longer period of deficiency was used so that the deficient animals were brought into estrus by a single injection of 10 units of PMS. All animals responded with an estrous vaginal smear and 55% of the group were successfully bred. Rats receiving no hormone injections during gestation showed a marked failure of implantation due to the longer period of deficiency prior to breeding; only 22% of the group implanted, in contrast to the usual 90–100% implantations, and all implantations resorbed. Rats injected with estrone and progesterone exhibited the usual favorable response, i.e., 100% implantations with 100% maintenance of pregnancy. Rats injected with the three pituitary gonadotrophins showed 78% implantations and partial maintenance of pregnancy; 29% of the group had living young. The marked improvement in implantation in contrast to the findings in the uninjected group shows the beneficial effects of injected gonadotrophins and indicates some pituitary dysfunction. Nevertheless, the failure to maintain pregnancy in a larger proportion of the animals would seem to clearly show ovarian dysfunction.

Whether the lack of ovarian response to injected gonadotrophins may be corrected by higher dosage levels, by a different hormone balance, or by supplementation with other pituitary hormones is not known. In adult rats hypophysectomized after breeding, daily injections of 60 I.U. MH and 60–200 μg. FSH-ICSH were successful in maintaining pregnancy (Lyons *et al.*, 1943; Lyons, 1951). In this study daily dosage levels of 120–200 μg. FSH-ICSH and 70–125 I.U. MH were used without the same degree of success. Although the hypophysectomized rat is considered to be more sensitive than an intact animal to pituitary hormones, it would usually be assumed that a vitamin-deficient animal with an intact pituitary would have some circulating gonadotrophin present. Such an animal would presumably require a decreased amount rather than an increased amount of gonadotrophin. Further studies on dosage levels are definitely indicated and are in progress.

The inadequate ovarian response to pituitary gonadotrophins brings up the question of possible placental dysfunction in these deficient animals. It was previously pointed out that although pregnancy could be maintained by estrone and progesterone in these deficient animals the number of living young per litter was less than half the normal value (Nelson, Lyons and Evans, 1951). The same results were obtained in this study, regardless of whether the injected hormones were ovarian or hypophysial in origin.

SUMMARY

Under the experimental conditions anterior pituitary gonadotrophins maintained pregnancy in approximately 25% of pyridoxine-deficient animals whereas ovarian hormones maintained pregnancy in 80–100% of such animals. This indicates that dysfunction of both the pituitary and the

ovary is involved in the hormonal inadequacies of these vitamin-deficient animals.

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THE PROGESTERONE CONTENT OF HUMAN PLACENTAL BLOOD[1]

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AN INVESTIGATION of the ketonic lipides of placental blood was undertaken in conjunction with our recent work (Pearlman and Cerceo, 1952a, 1952b) on the isolation of progesterone[2] and related C21 steroids from human placental tissue. In this study, however, progesterone could not be isolated because it was present in minute amount only. An estimate was made of the progesterone level of pooled placental blood by ultraviolet spectroscopy of the ketonic fraction following chromatography and countercurrent distribution.

EXPERIMENTAL

Collection, extraction, and preliminary fractionation of blood.—Full term placentas were obtained as soon as possible after parturition. Each specimen was perfused with 200 cc. of 0.9% NaCl through the umbilical vein following removal of clots of adhering maternal blood. The blood perfusate was immediately extracted and then partitioned in the manner outlined in Diagram 1 to yield the ketonic fraction.

Estimation of the progesterone content of the ketonic fraction.—The method used was that described by Pearlman and Cerceo (1952b) for estimation of the progesterone content of placental tissue[3] and requires measurement of the optical density at 240 mμ (maximal absorption due to α, β unsaturated ketones) following an 8 transfer distribution of the ketonic material between petroleum ether and 70% methanol; in the present study however, the volumes of the immiscible solvents were reduced to one-fifth.

The progesterone level of the blood perfusates of four placentas were individually estimated and found to fall within the range of 0.75 to 0.86 μg. of hormone per cc.; the pooled perfusates obtained from six other placentas were estimated to contain 0.69 μg. of progesterone per cc. These values are

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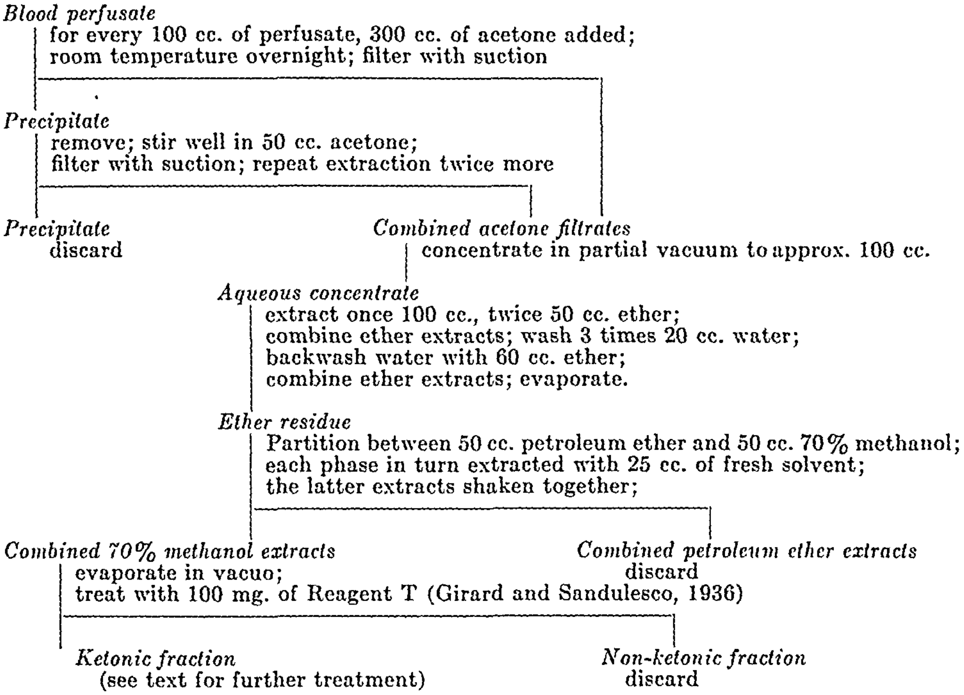
[2] Salhanick *et al.* (1952) have also announced the isolation of progesterone from human placenta; Diczfalusy (1952) simultaneously reported indirect evidence for the presence of this hormone.

[3] The procedure recently described by Diczfalusy (1952) is very similar but more refined, requiring a 24 transfer distribution between petroleum ether and 34.5% ethanol.

Diagram 1. Extraction and Fractionation of Placental Blood


Blood perfusate
for every 100 cc. of perfusate, 300 cc. of acetone added; room temperature overnight; filter with suction
Precipitate
remove; stir well in 50 cc. acetone; filter with suction; repeat extraction twice more
Precipitate
discard
Combined acetone filtrates
concentrate in partial vacuum to approx. 100 cc.
Aqueous concentrate
extract once 100 cc., twice 50 cc. ether; combine ether extracts; wash 3 times 20 cc. water; backwash water with 60 cc. ether; combine ether extracts; evaporate.
Ether residue
Partition between 50 cc. petroleum ether and 50 cc. 70% methanol; each phase in turn extracted with 25 cc. of fresh solvent; the latter extracts shaken together;
Combined 70% methanol extracts
evaporate in vacuo; treat with 100 mg. of Reagent T (Girard and Sandulesco, 1936)
Combined petroleum ether extracts
discard
Ketonic fraction
(see text for further treatment)
Non-ketonic fraction
discard

undoubtedly overestimated because the ketonic material in the peak tube (separatory funnel no. 4) in the countercurrent distribution did not give a well defined absorption curve characteristic of α, β unsaturated ketones; further purification of this fraction was needed.[4] Accordingly, the ketonic residues from separatory funnel no. 4, obtained in the countercurrent distribution of the above perfusates (total 1715 cc., ten placentas), were combined to yield 4.4 mg. of solids containing at the very most 384 µg. of progesterone. This material was dissolved in 0.2 cc. of dry ether, adsorbed on a narrow column containing 200 mg. of alumina[5] (Harshaw) previously wetted with the same solvent, and eluted with 20 cc. of dry ether. The eluate gave an ultraviolet absorption curve typical of an α, β unsaturated ketone, and was estimated to contain 186 µg. of progesterone;[6] counter-current distribution (Fig. 1) indicated a content of 182 μg. of progesterone (or of an α, β unsaturated ketone with a partition coefficient identical with that of progesterone). Recalculation, on the basis of the latter determination, of the progesterone level of the pooled blood perfusates (total 1715 cc.) gave a value of 0.39 μg. of the hormone per cc.

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[4] Reynolds and Ginsburg (1942) have similarly encountered pigments with nonspecific ultraviolet absorption which interfered with their attempts to estimate normal serum progesterone levels in another connection. In our experiments, such pigments were found to accumulate chiefly in the end separatory funnels in the countercurrent distribution procedure.

[5] The preparation is described in footnote 4 (Pearlman and Cerceo, 1952a).

[6] Chromatography of the same quantity of crystalline progesterone under identical conditions resulted in the recovery of 75% of the hormone according to measurements of the optical density at 240 mµ.

*The ketonic residues from separatory funnel no. 8 in the original counter*current distribution were similarly combined, representing the pooled perfusates from ten placentas. This material, from its position in the countercurrent distribution scheme, might be expected to contain α, β unsaturated ketones more polar than progesterone. However, the presence of interfering pigments could not be entirely eliminated by chromatography and further work on this fraction was abandoned.

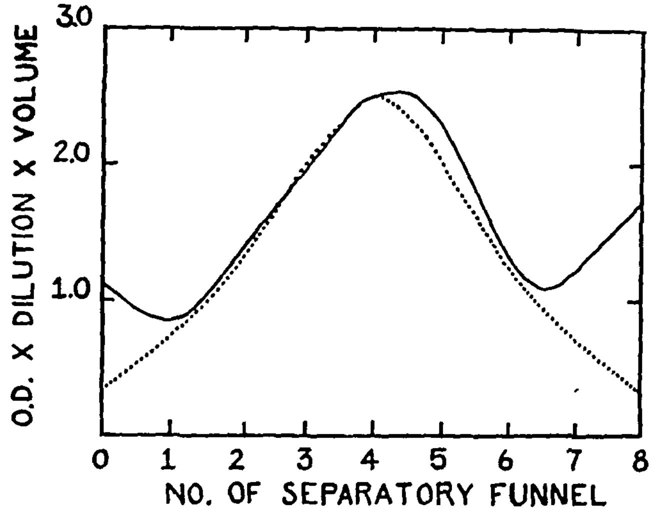

Fig. 1. Countercurrent distribution of chromatographed residue obtained from separatory funnel no. 4 (see text for further details). Solvent system, petroleum ether-70% methanol (volume ratio 3:1). Optical density (O.D.) measured at 240 mμ; solvent, absolute ethanol. Experimentally found curve, solid line; calculated curve for progesterone, dotted line.

DISCUSSION

The analytical procedures described herein were not designed for the routine examination of small specimens of blood, although these methods might be adapted for this purpose. Our aim, rather, was to obtain a sufficiently large quantity of placental blood (and hence resort to perfusion of the placenta) in order to carry out an exploratory examination of the ketonic material by physico-chemical means.

The data indicate that the progesterone concentration of pooled placental blood is of the order of 0.4 μg. of hormone per cc., a conservative estimate inasmuch as the blood obtained by perfusion underwent some dilution and also, part of the progesterone (roughly 25%) was lost by chromatography. It is clear that the placental blood could have contributed in small part only to the total progesterone content of our placental extracts (Pearlman and Cerceo, 1952b), a matter which was of some concern since, in that study, the blood had not been removed by perfusion of this highly vascular organ.

Although the absolute concentration of progesterone in placental blood is quite low, it is high by comparison with that in maternal blood, which is less than 0.1 μg. of progesterone per cc. (Butt *et al.*, 1951). The latter value, obtained by polarographic methods, is at variance with the higher values reported by Hooker and Forbes (1949) who employed bio-assay techniques.

A comparison of our data on placental blood with those of Butt *et al.* (1951) are of interest. The latter investigators found 1.3 and 0.3 μg. of progesterone per cc. in two specimens, respectively, of human placental blood obtained from the umbilical cord but in three other specimens, no progesterone (i.e., less than 0.1 μg.) could be detected; however, there was polarographic evidence for the presence of a Δ^4-3 ketosteroid but not of a 20-ketosteroid (the progesterone molecule contains both groups). The latter observation stimulated our interest but our study provides no information on this point.

SUMMARY

The progesterone level of pooled placental blood is conservatively estimated to be 0.4 μg. of hormone per cc. This calculation is based on measurements of the optical density at 240 mμ. of the ketonic fraction following chromatography and countercurrent distribution.

Acknowledgment

The authors are indebted to the Jefferson Medical College Hospital for permission to collect placental blood.

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DETECTABLE AMOUNTS OF ACTH IN BLOOD FOLLOWING SALICYLATE ADMINISTRATION

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ALTHOUGH a tabulation (Hailman, 1952) of various physiological responses induced by cortisone, ACTH, and salicylate (SAL) suggests an ACTH mechanism of SAL effect, an increase in circulating ACTH following SAL has not been demonstrated. Because of our general interest in the effect of salicylate upon adrenal function and the probability that some, at least, of these effects may be mediated through the pituitary gland we have undertaken to determine, by the transfusion technique of Farrell and McCann (1952), whether SAL administration stimulates release of ACTH into the circulation.

Female Sprague-Dawley rats (180–210 gm.) were injected intraperitoneally with 0.6 ml. of sodium salicylate solution containing the equivalent of 30 mg. of salicylic acid/100 gm. body weight. After varying intervals the rats were etherized and the external jugular vein quickly exposed. Three ml. of blood were withdrawn into a heparinized syringe and immediately transfused into the corresponding vein of an etherized, hypophysectomized Sprague-Dawley rat of approximately the same weight. This transfusion required about 1 or 2 minutes. The hypophysis had been removed from the recipient rat usually 2 days but not more than 3 days earlier. The left adrenal of the recipient rat was removed just before, and the right adrenal one hour after, the transfusion. Each adrenal was assayed for ascorbic acid without delay, using the method of Roe and Kuether (1942). As in the method of Sayers, *et al.* (1948), the difference in ascorbic acid concentration between the left and right adrenals of the recipient was taken as an index of ACTH level in the blood of the donor rat. The lowering of adrenal ascorbic acid is an effect thought to be specifically produced by ACTH under such conditions. To check both the effect of SAL itself and the operative procedures on the hypophysectomized rats, 0.2 ml. of 5.8% Na SAL was added to 2.8 ml. of blood obtained from donor rats injected 8–10 minutes earlier with isotonic saline solution instead of Na SAL; and this blood mixture was assayed similarly for ACTH activity by injection into control hypophysectomized rats.

SAL levels and adrenal ascorbic acid depletion in the donor rats were

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TABLE 1. ADRENAL ASCORBIC ACID (AAA) DEPLETION IN HYPOPHYSECTOMIZED RATS ONE HOUR AFTER TRANSFUSION OF 3 ML. OF BLOOD FROM SAL-INJECTED RATS

Time after SAL injection of donor rats	No. of recipient rats	Decrease in AAA in hypophysectomized recipient rats	
minutes		mg./100 gm. of adrenal tissue	%
control	7	7 ±20*	2
5	2	−3	0
15	8	50 ±10*	12
30	3	38	10
60	4	31 ±17*	8
85	2	12	3

* ± values are standard errors, calculated where sufficient numbers of animals were used for significant statistical treatment.

determined in order to correlate them with the adrenal ascorbic acid reduction in the recipient rats. These were compared also with control donors receiving isotonic saline instead of SAL injections. The method of Smith and Talbot (1950), involving the use of the Folin-Ciocalteu reagent for phenols, was used in the estimation of the plasma SAL levels.

RESULTS AND DISCUSSION

The dosage of SAL used was about $\frac{1}{2}$ of the lethal dose (Waddell, 1911). After injection the rats showed no obvious discomfort and exhibited no unusual behavior. The rats were gentle and exhibited very little excitement when injected, thus minimizing any ACTH rise resulting from the mere handling of the animals.

The results presented in Tables 1 and 2 indicate that very soon after the SAL is injected, there is an increase in circulating ACTH as reflected by a decrease in both donor and recipient adrenal ascorbic acid. Since injected ACTH is rapidly destroyed, the results in terms of percentage decrease in adrenal ascorbic acid are less striking in the recipients than in the donors. Despite the continued high blood level of SAL in the donor the

TABLE 2. ADRENAL ASCORBIC ACID (AAA) AND PLASMA SAL LEVELS IN DONOR RATS INJECTED WITH 30 MG. SAL/100 GM. BODY WEIGHT

Time after injection of donor rats	Type of injection	No. of donor rats	AAA	AAA decrease	Donor plasma SAL levels minus blank
minutes			mg./100 gm. of adrenal tissue	%	mg./100 ml.
15	SAL	10	375	19	57.6
15	Saline	5	463		0
60	SAL	5	232	50	55.6
60	Saline	5	463		0
120	SAL	5	158	54	40.6
120	Saline	5	347		0

ACTH activity appears to decline from an initial maximum, presumably as the preformed donor ACTH stores are consumed.

SUMMARY

The ACTH titer in blood of SAL-injected rats has been shown to rise very soon after the injection, as assayed by transfusion into hypophysectomized rats of blood from SAL-injected rats.

Acknowledgment

The advice of Dr. J. S. Davis on surgical procedure is gratefully acknowledged. The authors also wish to thank the S. E. Massengill Co., of Bristol, Tennessee, for providing some of the animals and chemicals used here. The hypophysectomized rats were obtained from Hormone Assay Laboratories, Chicago, Illinois.

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GLUCURONIDASE HYDROLYSIS FOR PREGNANEDIOL ASSAYS[1,2]

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THE great interest shown in pregnanediol assays is based upon a proof of relationship between pregnanediol and the corpus luteum hormone —progesterone (Marrian 1949). The methods for assaying pregnanediol in urine are: Firstly the method of Venning (1937, 1938) in which the sodium pregnanediol glucuronidate (NaPG) of the urine is assayed gravimetrically; and secondly the hydrolysis of the pregnanediol conjugate and the assay of the pregnanediol (Astwood and Jones, 1941; Talbot *et al.*, 1941). In 1948 Sommerville, Gough, and Marrian modified the test in order to obtain a sensitivity of 2.5 mg./day. This paper introduces the hydrolysis of urines by calf spleen glucuronidase. This method has been found to give excellent results for assaying less than 0.50 mg. pregnanediol /24 hrs.

MATERIALS

All chemical reagents were chemically pure. The toluol was redistilled. The sodium pregnanediol glucuronidate was freed of sodium pregnaneolone glucuronidate by the procedure of Sutherland and Marrian (1947). An all glass filter funnel similar to that described by Engley (1952) and containing a sintered glass filter of medium porosity was used to filter off the pregnanediol before assay. The glucuronidase was prepared and assayed according to the procedure of Cohen (1951). The normal male urine used in these experiments was pooled urine. The pregnancy urines were collected from individual girls from 32 to 38 weeks of pregnancy. The pregnanediol assays with sulphuric acid were carried out essentially by the procedure of Talbot *et al.* (1941).

EXPERIMENTAL

The Hydrolysis of NaPG and the Purification of Pregnanediol

The technique described by Cohen (1951) for the hydrolysis of pregnanediol glucuronide was employed. A modified technique of Sommerville

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[1] This work was aided by grants from the National Institutes of Health, United States Public Health Service and from the Graduate School of the University of Minnesota.

[2] This paper was read before the Endocrine Society on June 5, 1952.

[3] The work reported herein is taken largely from a thesis being presented by M. M. Goldfine in partial fulfillment for the degree of Master of Arts by the University of Minnesota Graduate School.

et al. (1948) was employed for the purification of pregnanediol. This scheme is outlined in Chart I.

RATE OF HYDROLYSIS

The hydrolysis of late pregnancy urine gives the same type of curve as did that employing pure NaPG (Cohen 1951). Hydrolysis is complete within 5 to 6 hours, indicating that urine NaPG is about twenty times more labile to glucuronidase hydrolysis than are most steroids. These results are illustrated in Figure 1.

CHART I. Procedure for the hydrolysis of NaPG and purification of urinary pregnanediol.

URINE
100–500 cc.
- *Sterilize by boiling 10 minutes*
- Cool, adjust to pH 4.5 (0.1 N) with acetate buffer +1000 units glucuronidase, incubate 6 hours to overnight
- Extract with 2×1/2 vols. toluene (emulsions broken by configuration)

——————————URINE RESIDUE

TOLUENE
- Wash with 2×1/10 vol. N. NaOH. 1×1/10 vol. H_2O
- To dryness *in vacuo*
- Dissolve residue in 15–20 cc. hot ETOH, +1–2 mg. charcoal; filter (Whatman 12.5 cm. No. 12 Filter Paper)

——————————CHARCOAL

FILTRATE
- Concentrate to 5 cc. volume; +20 cc. hot 0.1 N NaOH. and incubate at 37° for 2–4 hrs. Filter (Engley funnel)

——————————FILTRATE

PRECIPITATE
- Dissolve in 15–25 cc. ETOH

ASSAY ALIQUOTS

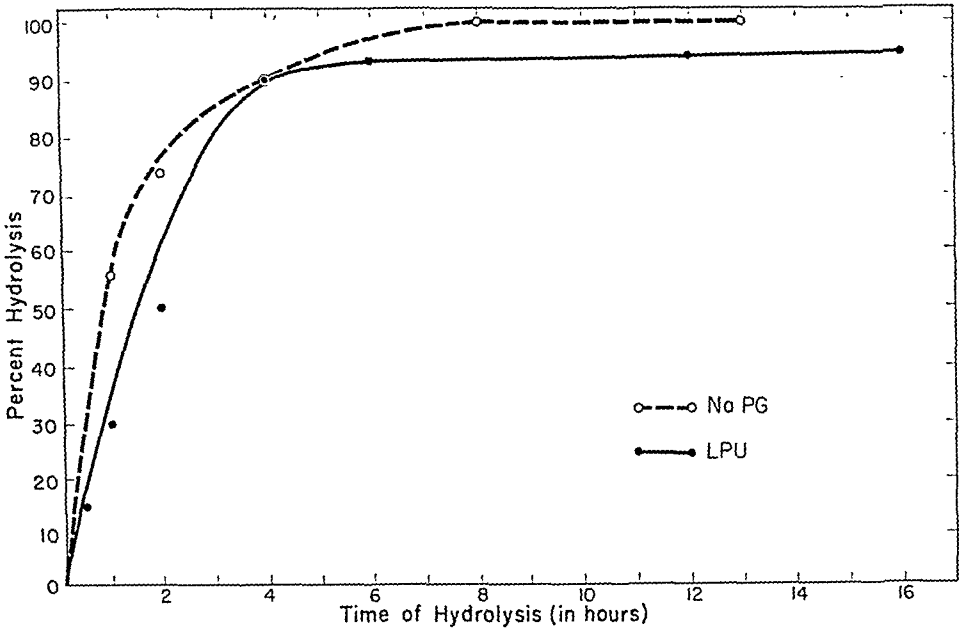

FIG. 1. The hydrolysis of pure NaPG and of late pregnancy urine (L.P.U.) with 1000 units glucuronidase.

RECOVERIES OF PURE NaPG ADDED TO MALE URINE

The data outlined in Table 1 illustrates that good recoveries can be obtained down to 0.04 mg. of pregnanediol per 100 cc. urine.

RECOVERIES OF PREGNANEDIOL FROM HUMAN PREGNANCY URINE

In Table 2 the accuracy of the pregnanediol assay is further shown. It can be seen from this table that glucuronidase liberates as much preg-

TABLE 1. AMOUNT OF PREGNANEDIOL RECOVERED FROM MALE URINE TO WHICH NaPG HAD BEEN ADDED BY GLUCURONIDASE (Gl) HYDROLYSIS

Pregnanediol added as NaPG	Solvent	Gl hydrolysis (corrected for control)
3.40 mg.	100 cc. NMU P_1	3.23
1.70 mg.	100 cc. NMU P_1	1.65
control	100 cc. NMU P_1	0
0.65 mg.	100 cc. NMU P_2	0.52
0.33 mg.	100 cc. NMU P_2	0.31
control	100 cc. NMU P_2	0
0.26 mg.	100 cc. NMU P_3	0.25
0.104 mg.	100 cc. NMU P_3	0.101
0.052 mg.	100 cc. NMU P_3	0.060
0.039 mg.	100 cc. NMU P_3	0.030
0.013 mg.	100 cc. NMU P_3	0.022
control	100 cc. NMU P_3	0.030

TABLE 2. COMPARISON OF PREGNANEDIOL RECOVERED FROM PREGNANCY URINE AFTER HYDROLYSIS BY HCl AND BY GLUCURONIDASE

Patient	Volume urine assayed	Pregnanediol recovered hydrolysis with	
		HCl in mg./24 hrs.	Glucuronidase in mg./24 hrs.
D. R.	100 cc.	36.8	34.2
R. A.	100 cc.	61.2	64.0
H. G.	100 cc.	72.0	73.1
M. S.	100 cc.	58.0	58.0
H. M.	100 cc.	32.0	32.6
L. M.	100 cc.	42.0	43.0
J. S.	100 cc.	44.0	45.0

nanediol as does HCl from 100 cc. pregnancy urine. Table 3 illustrates that pregnancy urine can be diluted so that 2 cc. of urine containing 0.14 mg. of pregnanediol can be readily assayed. In no case did an HCl hydrolysis effected after the glucuronidase hydrolysis yield more than the equivalent of 1 mg. pregnanediol per 24 hours. It would thus seem that in pregnancy urine at least, all of the pregnanediol is present as the glucuronide.

APPLICATION OF GLUCURONIDASE HYDROLYSIS TO STUDIES OF THE NORMAL FEMALE CYCLE

In Figure 2 is shown the level of pregnanediol excretions for a number of normal women. In these experiments assays were conducted on 500 cc. batches of urine. It will be noted that pregnanediol excretion shows a gradual increase during the follicular phase of the menstrual cycle. It does not reach its peak until a week or so before the next menses. It then falls rapidly so as to reach low levels by the time menses have begun. It will be noted that for patient K. W. the pregnanediol reached an excretion level of about 1.5 mg. per diem at the usual time but then fell abruptly, and did not rise again (to 2.5 mg. per day) until the 39th day of the cycle. As a result this girl had a 42 day cycle.

It will be noted (Fig. 2) that melting points carried out on a number

TABLE 3. EFFECT OF DILUTION OF PREGNANCY URINE (TO 100 CC. WITH H_2O) ON PREGNANEDIOL ASSAY (PATIENT D.P.).

Volume urine assayed	Theoretical, mg. pregnanediol	Actually found, mg. pregnanediol
100 cc.	6.9	6.9
50 cc.	3.5	3.5
25 cc.	1.7	1.8
10 cc.	0.69	0.75
5 cc.	0.34	0.34
2 cc.	0.14	0.14

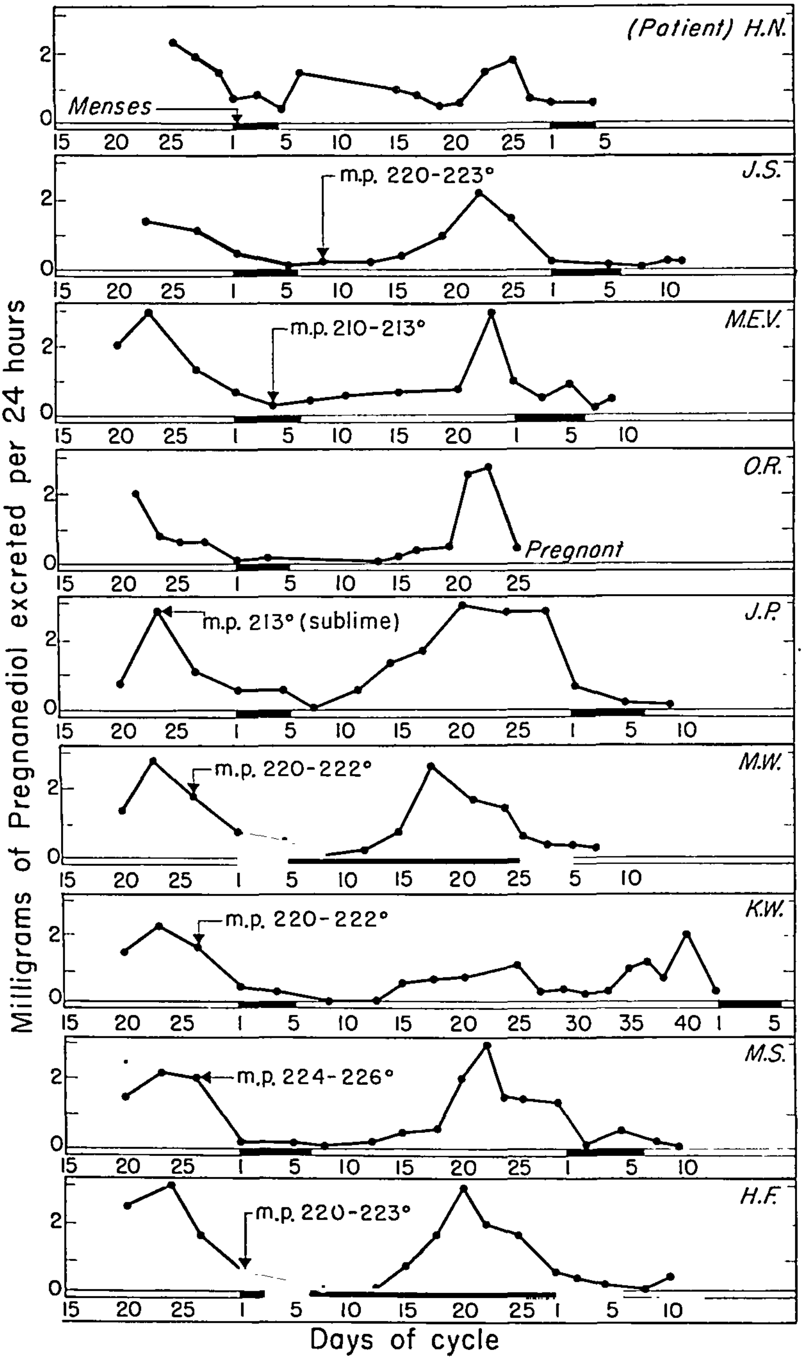

FIG. 2. The excretion of pregnanediol during the menstrual cycle.

of residues varied from 210° to 224° (pure pregnanediol melts at 236°–237°, uncorrected).[4]

THE EXCRETION OF PREGNANEDIOL DURING EARLY PREGNANCY

Guterman (1944, 1945) reported on the use of a pregnanediol test for the detection of pregnancy, a finding confirmed by one worker (McCormack 1946) and criticized by a number of workers (Semmens and McHenry, 1949; Kullander, 1950; Merivale, 1948; Reinhart and Barnes 1946). In Table 4 are shown some results which we have obtained using the glucuronidase hydrolytic procedure. Since in the normal cycling women we found no pregnanediol assay greater than 3 mg./24 hours it is of interest to point out that within 1 day of the missed menses of patient D a level of 3.3 mg./24 hours and for patient R on the 5th day following the missed menses, 4.0 mg. pregnanediol was excreted. By the 4th week after the first missed menses the excretion of pregnanediol was at least 5 mg./24 hours. It is of interest to point out that patient Y, with an excretion level of only 4.2 mg. pregnanediol/24 hours in the 16th week of pregnancy aborted shortly thereafter. For patient E. H. the first urine sample collected 13 weeks after the first missed menses the pregnanediol excreted was relatively low. This patient had aborted in an earlier pregnancy and was feeling quite ill (malais and vomiting) at the time this specimen was collected. Four weeks later this had passed and the pregnanediol secretion had risen to 17.4 mg./day.

TABLE 4. PREGNANEDIOL EXCRETION BY EARLY PREGNANCY CASES[1]

Patient	*Approximate time* lapsing since 1st missed menses	mg. pregnanediol /24 hrs.	Comment
D	1 day	3.3	
D	4 weeks	7.2	
O.R.	1 week	4.0	
O.R.	17 weeks	9.8	
A	4 weeks	5.5	
Z	4 weeks	6.5	
P	5 weeks	6.5	
E.Z.	10 weeks	15.4	
S	12 weeks	7.2	
B.H.	12 weeks	6.9	
E.H.	13 weeks	4.8	Had a previous abortion
E.H.	17 weeks	17.4	
X	14 weeks	16.1	
Y	16 weeks	4.2	Aborted 1 week later
O	18 weeks	12.5	

[1] We would like to thank Dr. I. H. Kaiser of the Department of Obstetrics and Gynecology, University of Minnesota Hospitals, for aid in collecting some of these urines.

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[4] The melting points were determined on a Fischer micro melting point apparatus and are uncorrected.

DISCUSSION

The use of glucuronidase as a hydrolytic means for pregnanediol in urine offers the following advantages: firstly, the conditions for effecting a complete hydrolysis with glucuronidase of NaPG results in very little hydrolysis of any other steroid glucuronide. This is true because of the use of an overnight incubation with 1,000 units of enzyme per 500 cc. urine (most conjugated steroids require an incubation of 3 to 4 days with 5,000 units per 25 cc. urine) and because the optimum pH for glucuronidase activity for NaPG is 4.5 while most of the steroid glucuronides have an optimum pH of 5.0 (Cohen, 1951). Secondly, the test is more sensitive than any other yet described, since 0.5 mg. of pregnanediol/24 hours can be accurately assayed. Apart from the time required for incubation about 6 assays can be run per working day.

Our figures for pregnanediol assays during the cycle are about one-half those reported by workers assaying for NaPG (Venning and Browne, 1937; Hain and Robertson, 1939; Buxton, 1940). This is not surprising in view of the reports by Marrian and Gough (1946) that the NaPG isolated according to the procedure of Venning and Browne (1936) contains 23% pregnaneolone. Smith and Smith (1952) have reported the presence in NaPG of up to 20% of some additional as yet not identified steroid glucuronides. Since our assay technique is a quantitative one we feel that it may offer an excellent diagnostic tool for pregnancy and for gynaecological disturbances.

SUMMARY

The glucuronidase hydrolysis has been adapted for the assay of pregnanediol in urines, by introducing a relatively simple purification procedure. This test is highly sensitive, gives material with a good melting point and offers a means of hydrolysis whereby other steroids are hydrolyzed to only a minor degree. It has been found possible to get effective assays on urines collected from girls in the early follicular phase of the cycle. It has also been found possible to utilize the assay procedure in the diagnosis of pregnancy.

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NOTES AND COMMENTS

GONADOTROPINS OF THE PITUITARY GLAND DURING INFANCY AND EARLY CHILDHOOD

The purpose of this note is to present data on the gonadotropins of the pituitary gland of 2 infants and 1 child.

I. MATERIALS AND METHODS

A. Animals.—Female Wistar rats (30 to 40 days old) were hypophysectomized, injected with pituitary homogenate and subsequently studied as described previously (Bahn and associates, *1953* a, b).

B. Pituitary Tissue.—The pituitary glands of 2 infants, 1 male and 1 female, and 1 male child, 4 years old, were obtained within three to four hours following death. No prolonged terminal illness was present in any of the 3 patients. The wet weight of the combined anterior lobes of the hypophyses of the infants was 136 mg., and that of the child, 222 mg. The effects of total doses of only 10 mg. and 30 mg. of the gland of the infants and only 5 mg. and 30 mg. of that of the child were determined owing to the limited amount and scarcity of tissue.

II. RESULTS

A. Homogenate of Infants' Hypophyses.—The data in Table 1 indicate that no follicle-stimulating hormone (FSH) and no luteinizing hormone (LH, ICSH) activity was present in 30 mg. of pituitary homogenate.

B. Homogenate of the Child's Hypophysis.—Both FSH and LH activity were detected (Table 1). Marked follicular growth accompanied by ovarian enlargement was observed at a dose of 30 mg. Although complete repair of the interstitial cells was not observed, the uterine enlargement indicated estrogen secretion and thus the presence of LH. In terms of the previously defined units, there was somewhat more than 1 unit of FSH but less than 1 unit of LH per 30 mg. of pituitary homogenate.

TABLE 1. RESPONSES TO PITUITARY HOMOGENATE

Animals		Homogenate		Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Source	Dose, mg.	mg.	mg./gm.	mg.	mg./gm.	F.D.	C.L.	I.T.	
Hyp.	6	—	0	6.5	0.11	22.7	0.39	0	0	D	0
Intact	4	—	0	32.8	0.28	167.5	1.44	+++	+	R	+
Hyp.	2	I	10	4.1	0.07	18.6	0.30	0	0	D	0
Hyp.	2	I	30	8.1	0.10	30.6	0.36	0	0	D	0
Hyp.	4	C	5	8.7	0.12	24.6	0.33	0	0	D	0
Hyp.	2	C	30	13.4	0.18	99.9	1.31	+++	0	D	+

Abbreviations: Hyp. = Hypophysectomized; mg./gm. = Organ weight per gram body weight; D = Deficient; R = Repair; I.T. = Interstitial tissue; 0 = Absent; + = Present; V.O. = Vaginal opening; F.D. = Follicular development; I = Pooled infants' hypophyses; C = Child's hypophysis; C.L. = Corpus luteum.

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While the organization of this book does not permit any special emphasis on the endocrine glands, it must be apparent that modern endocrine histophysiology depends increasingly on histochemical techniques. Pearse's *Histochemistry* should prove a valuable addition to the library of any endocrinologist who intends either to apply histochemical methods or to interpret the significance of his own or others' results.

HELEN WENDLER DEANE

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THE BIOASSAY OF GLUCO-CORTICOID ACTIVITY BY THE MOUSE GLYCOGEN METHOD: SOME NEW FACTS

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EXPERIENCE with the mouse glycogen method for the bioassay of gluco-corticoid activity was obtained while investigating the conversion of steroids by adrenal tissue. A number of new facts emerged, some of which of primary importance in the assessment of the accuracy and merit of different bioassay procedures. The method of Venning *et al.* (1946) measures the deposition of glycogen in the depleted livers of fasted adrenalectomized mice, whereas the methods of Eggleston *et al.* (1946) and of Dorfman *et al.* (1946) depend on maintaining the well-stocked depots of hepatic glycogen during fasting. Venning's method appeared to possess greater accuracy, and was adopted for the present investigation.

METHODS

Operative technique and anaesthesia. The adrenals were removed by the lumbar approach, at first under pentobarbitone-ether anaesthesia, but better results were obtained with combined local and intraperitoneal xylocaine. The intraperitoneal dose produced anaesthesia of the peritoneum, as well as a state of stupor during which the operation was carried out. Mice weighing 20–26 g. received 0.15 ml. of 1% xylocaine without adrenaline subcutaneously and 0.15 ml. intraperitoneally. For mice under 20 gm., the intraperitoneal dose was 0.1 ml. Half the subcutaneous dose was placed over the right lumbar peritoneal incision and half over the left, and the operation performed without pain response 5–10 minutes after the injection of xylocaine. The tail of the mouse was fixed by adhesive plaster, and its right ear held by an assistant. The animals suffered less discomfort and shock than with ether. By the fourth postoperative day, the mortality for 250 mice adrenalectomized under pentobarbitone and ether was 20%, while the mortality for 1682 mice adrenalectomised under xylocaine was 6.9%.

Strain of mice. Venning *et al.* used pure strain mice from their "own colony" but in the present work reliance had to be placed on dealers for the uniformity of the strain of mice.

The "constant temperature room." Venning *et al.* kept their adrenalectomized animals in a "constant temperature room" at 76° F. In the present work, the temperature was

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kept above 70° F. by a thermostat, but there were no means of reducing high temperatures in the summer. The temperature of the animal room was recorded three times daily, however, and a study made of the effects of its variation.

RESULTS

Preliminary assays on adrenal cortical extract, *Eucortone,* showed marked individual variation. This variation was particularly noticeable in the 20 control animals, four of which gave values over 10 mg. glycogen per 100 gm. body weight, *viz.,* 26.3, 28.5, 66.7 and 67.2. The mean and its standard error were 10.86 ± 4.64, while the figures of Venning *et al.* (34 controls) were 4.1 ± 0.5, showing a marked difference in the mean, and a more striking difference in its standard error. Postmortem failed to reveal remnants of glands or accessory adrenal rests. The causes of this high variation were next investigated.

Liver glycogen levels in normal and in fasted non-adrenalectomized mice. The standard error of the mean liver glycogen (Table 1) reveals again wide variation in all four groups, a variation which in this case could not be ascribed to faulty operative technique.

TABLE 1. LIVER GLYCOGEN LEVELS IN NORMAL NON-FASTED MICE, AND IN NORMAL MICE FASTED FOR DIFFERENT PERIODS

Group	Number of animals	Period of fast in hours	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation
a	30	0	81.08 ± 11.53	77.9
b	10	8	40.62 ± 10.12	78.8
c	30	24	16.80 ± 3.7	120.5
d	20	24	24.35 ± 5.91	108.5

The coefficient of variation was found useful, particularly for comparing the present results with those of other workers, and led to interesting conclusions. In biological measurements, coefficients greatly exceeding 25% indicate a multiplicity of variables and necessitate the use of large numbers of animals in assays. A study of the factors concerned with the object of reducing the coefficient of variation is then desirable, and may result in a considerable increase in the accuracy of such assays.

The coefficient of variation of the first 20 control adrenalectomized mice was 191.2, while the coefficients for normal and fasted normal mice are shown in Table 1. An inverse proportionality is noted between the mean and its coefficient of variation in the latter group, so that the higher coefficient of the adrenalectomized controls could be attributed to a lower mean. A later group of starved *normal* mice gave a mean liver glycogen of 8.4 and a variation coefficient of 218.56. The technique of adrenalectomy was evidently exonerated.

The precision of the chemical estimation of liver glycogen was satisfactory as determined by control experiments in which known amounts of glycogen were added to pieces of liver.

Stomach contents of fasted mice. Some fasted mice consumed large quantities of the sawdust bedding contained in their cages. When sawdust was excluded, the mean liver glycogen was lower, the "t" test giving P <0.001.

TABLE 2. LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE

Group	Number of animals	Liver glycogen per 100 gm. mouse mean ±S.E.	Coefficient of variation
1	10	4.679	
2	10	29.166	
3	10	14.309	
4	10	26.470	
5	10	7.210	
6	10	6.830	
7	10	8.628	
8	10	25.590	
9	10	9.328	
10	10	2.026	
11	10	9.498	
12	10	19.474	
13	10	5.475	
14	10	14.925	
15	10	2.781	
1–15	150	12.4±1.8	178.0

TABLE 3. COMPARISON OF LIVER GLYCOGEN LEVELS IN FASTED ADRENALACTOMIZED CONTROL MICE KEPT ON DIFFERENT DIETS DURING THE PERIOD OF PREPARATION

Group	Number of animals	Diet	Liver glycogen per 100 gm. mouse mean ±S.E.	Coefficient of variation	*t*	*P*
a	150	stock diet	12.4 ±1.8	178.0	—	—
b	61	diet A+B	12.66±2.93	181.25	0.077	>0.9
a+b	211	as above	12.48±1.53	178.5	—	—

The coefficient of variation remained very high, however, so that sawdust eating contributed only a little to the scatter of the results.

Fasted mice ate cotton wool, faeces, their own fur and the bodies of dead mice in their cage. In all subsequent experiments mice were kept in special meshwire cages without bedding and with no access to each other. The high coefficient of variation was hardly affected, however, No correlation was established between liver glycogen and gastro-intestinal contents at the time of killing.

Diet and liver glycogen. The 34 control adrenalectomized mice of Venning *et al.* (1946) fasted for 22½ hrs., gave a mean liver glycogen of 4.1 mg./100 g. No mention was made of gross deviations, nor are they suggested by the small standard error (0.5). The mean liver glycogen of the 242 control mice of Dorfman *et al.*, fasted only $6\frac{1}{2}$ hrs., was 10.8. Large variations were mentioned, some of the control groups averaging as much as 31.5 mg./100 gm. The 28 control mice of Eggleston *et al.* (1946) also fasted for $6\frac{1}{2}$ hrs., averaged less than 5 mg./100 g. and wide variations were not described. In the present investigation, the mean of 150 mice, fasted for $22\frac{1}{2}$ hrs., was 12.4 ± 1.8 with a variation coefficient of 178.0 (Table 2).

Forbes *et al.* (1950) published results of assays with a modified form of Eggleston's method. When mice were fed on the McCollum diet (Diet A), "positive controls," i.e. mice showing more than 9 mg. liver glycogen per 100 gm., occurred frequently. The substitution of bran for whole wheat flour in the McCollum diet (Diet B) resulted in more "positive controls," with values as high as 179.4 mg./100 gm. When "Diet B" was substituted for "Diet A" in the last 15 hrs. before the fast, however, "more rapid emptying of the intestinal tract occurred," and only one "positive control" encountered in a group of 34.

Combined "Diet A+B" was therefore tried in 61 mice, and the results compared with the 150 control mice on stock diet (Table 3). There is no significant difference in the mean or variation coefficient, and the "t" test gives $P > 0.9$.

Liver glycogen levels in control mice do not follow the normal distribution, a fact which may vitiate the application of the "t" test to the two groups. The *means* of samples from abnormal distributions, however, rapidly conform to the normal curve with increase in the size of sample (Emmens, 1948; Finney, 1950). A comparison of the two control groups, based on the mean values of subgroups of 10 mice, is given in Table 4. The "t" test again gives $P > 0.9$.

Temperature and liver glycogen. During the hot summer months, assays gave high results, and liver glycogen in control mice showed striking rises which necessitated the rejection of previous experiments. The effect of temperature was important not only on the day of test but throughout the postoperative period. Temperatures of 76–80°F with little effect in dry weather produce a marked increase in liver glycogen if the atmosphere is humid (Table 5).

Experiments carried out with the object of reducing the error of the assay. Attempts were made to reduce liver glycogen by a variety of procedures in the hope of eliminating "positive controls," even though at the expense of some loss in sensitivity. Forced exercise for 4 hrs.—by the rotating drum method—prior to the assay, and exposure to 4°C for the same period, did not produce the desired result. The possibility of a glycogenic steroid being evolved by the gonads was investigated by performing combined orchidectomy and adrenalectomy on the test mice, but this also gave no better results. A third method was yet tried. Three groups of mice were given daily subcutaneous injections of 10 g. of l-thyroxine sodium for 4–5 days up to the day of assay. This succeeded in eliminating control values over 10 mg./100 gm. (mean 3.33 ± 0.81), but the coefficient of variation was still unduly high (132.5%), and mice were rendered totally insensitive to Eucortone.

Assay of cortisone. Failure to secure low liver glycogen in control mice and uniform response in mice injected with gluco-corticoids was persistent. It was therefore decided to proceed with the investigation originally planned, employing for the assay the only mice that were available. An assay was carried out with cortisone acetate as a standard (Table 7). A large number of animals, *viz.*, 40 for each dose, had to be used to obtain a reasonably small standard error, and 20 μg represented the smallest

TABLE 4. COMPARISON OF LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE KEPT ON DIFFERENT DIETS DURING THE PERIOD OF PREPARATION

(Statistics based on mean values of groups, each consisting of about ten mice)

Group	Number of animals	Diet	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	*t*	*P*
a	150	stock diet	12.43 ± 2.3	71.75	—	—
b	61	diet A + B	12.61 ± 2.37	46.0	0.0454	<0.9
a + b	211	as above	12.48 ± 1.80	64.75	—	—

TABLE 5. COMPARISON OF LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE UNDER DIFFERENT WEATHER CONDITIONS

Group	Number of animals	Temperature range ° F.	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	*t*	*P*
a	211	74 ± 4	12.48 ± 1.53	178.5	—	—
b	91	80 ± 4	70.13 ± 8.03	109.75	9.975	<0.001

TABLE 6. LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED CONTROL MICE SUBJECTED TO VARIOUS TREATMENTS DURING THE PERIOD OF PREPARATION

Group	Number of animals	Treatment	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	*t*	*P*
a	211	nil	12.48 ± 1.53	178.6	—	—
b	9	cold—4° C. for 4 hours	9.42 ± 5.43	173.0	0.403	>0.6
c	11	forced exercise	20.50 ± 9.14	147.75	1.116	>0.2
d	9	orchidectomy	37.72 ± 12.27	97.5	—	—
e	30	thyroxine	3.33 ± 0.81	132.5	2.167	<0.05

TABLE 7. LIVER GLYCOGEN LEVELS IN FASTED ADRENALECTOMIZED MICE INJECTED WITH CORTISONE ACETATE ON DAY OF TEST

Group	Number of animals	Dose in μg.	Liver glycogen per 100 gm. mouse mean ± S.E.	Coefficient of variation	t	P
a	211	0	12.48 ± 1.53	178.5	—	—
b	20	10	17.33 ± 9.13	235.0	0.850	>0.3
c	40	20	28.08 ± 5.33	120.0	1.086	>0.2
d	40	40	52.08 ± 9.54	116.0	2.184	<0.05
e	40	80	105.70 ± 14.12	84.5	3.148	<0.01

amount of cortisone capable of producing a significant difference in response as seen from the "t" test between doses.

The coefficient of variation is again persistently high, though it diminishes in an inverse manner with increase in the mean. A group of fasted *normal* mice injected with 0.1 ml. *Eucortone* and showing a mean of 46.83 had the same coefficient of variation (116) as the group of adrenalectomized mice injected with 40 μg cortisone with a mean of 52.08. Thus, animals showing comparable means have comparable coefficients of variation in both normal and adrenalectomized groups. This relationship emphasizes again the independence of the high scatter from the technique of adrenalectomy.

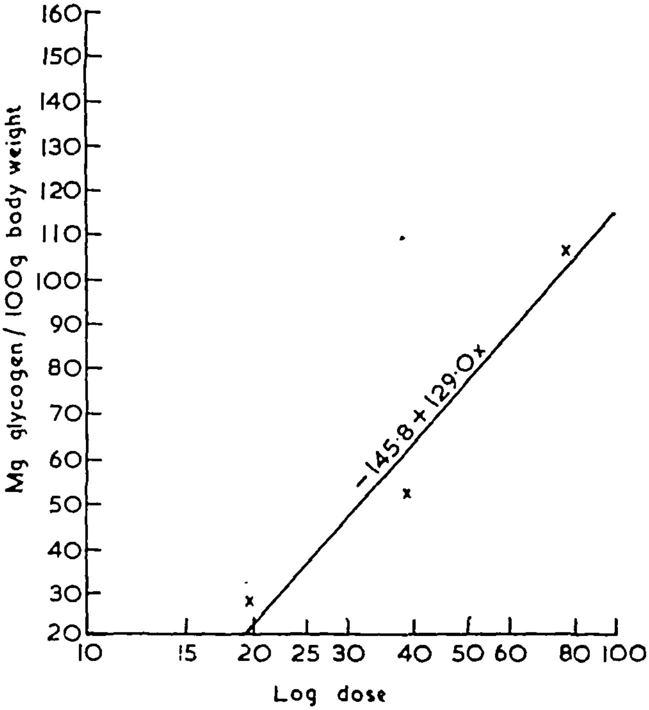

FIG. 1. Logarithm dose-response regression line for cortisone acetate—from 3 points, 20, 40 and 80 micrograms.

The responses to the last three doses of cortisone show a reasonably good fit to a log-dose repsonse curve (Fig. 1). If the smallest dose (10 μg) is included, the fit is impaired considerably. The reason for this is understood from Figure 2, where all four points are plotted as a linear dose-response curve and fit the regression line almost perfectly. Regression lines are often linear in the lower range of doses of an active substance, and this appears to be the case with cortisone acetate over the range 10–80 μg. The log-dose response curve is used in the comparative studies which follow.

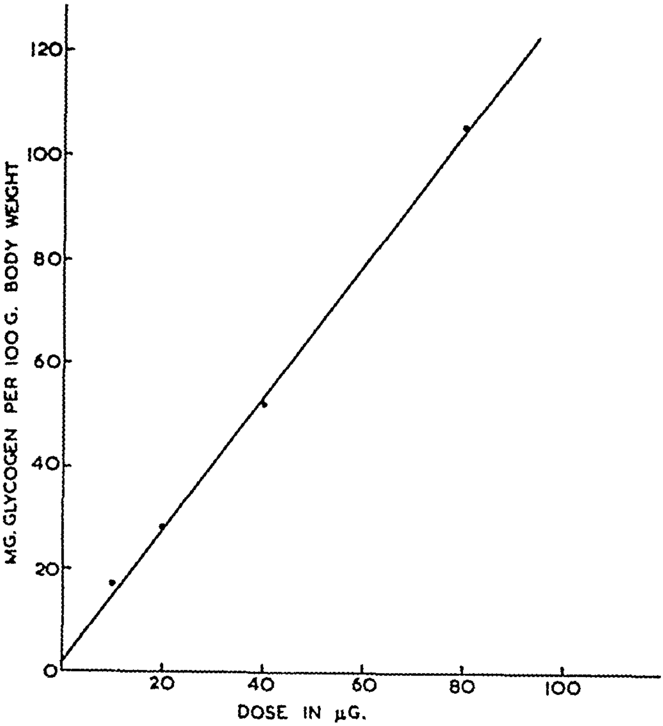

FIG. 2. Linear dose-response regression line for cortisone acetate—from 4 points, 10, 20, 40 and 80 micrograms.

DISCUSSION

The findings of the present investigation are different in several respects from those of other workers, and their careful consideration may result in the saving of time and trouble for many an investigator. These findings have also prompted the critical analysis of the data and conclusions of other authors.

Temperature and liver glycogen. Venning *et al.* (1946) found that during the summer months the "standard dose of 70 mg glucose will cause some deposition of glycogen and consequently the deposition values for known amounts of adrenal corticoid are greater." They recommended that the sensitivity of mice be checked from time to time with a crystalline gluco-corticoid hormone. The present results indicate that the matter is more serious. Liver glycogen levels in fasted non-adrenalectomized mice which *have not received glucose* show that in hot weather the hepatic glycogen depots are more richly stocked and more resistant to depletion. Many mice show glycogen levels over 200 mg/100 gm without the help of glucose. Unless the animal room is equipped with an automatic cooling device, bioassay experiments must be interrupted during hot weather. A check on sensitivity with a crystalline gluco-corticoid may not be sufficient, since variation among the two groups may be great enough to lead to fallacious conclusions.

The temperatures at which various authors kept their mice were different. Venning *et al.* kept their mice at 76°, Eggleston *et al.* at 78° and Forbes *et al.* at 80 ± 3° F. In the present work, temperatures in the range used by Forbes *et al.* gave high control values (Table 5). The range of 74 ± 4° F. was found to give reasonably reliable results. Whether these differences are ascribed to differences in strain of mice or to other climatic factors, e.g. humidity, it is evident that each investigator will have to find his own optimum range of temperature.

Comparative study of the coefficient of variation. At first the widely scattered values of liver glycogen in control and injected animals were bewildering. Control experiments removed suspicion from the operative procedure and chemical estimation of liver glycogen. With the progress of the work, it became clear that the high coefficient of variation was a characteristic of the mice used. Except for some inverse proportionality with the mean, the magnitude of this coefficient changed little throughout all experiments and was independent of diet, temperature variation, body weight and amount of body fat. To all appearances, therefore, the high coefficient of variation is a constant characteristic of the colony of mice, and is most probably an index of the admixture of strains in that colony.

The above argument is strengthened by the results shown in Table 8, where a comparative study is made of the coefficients of variation of the present experiments and those of other workers. The coefficient of variation is highest in control groups and mostly shows gradual dimunition with increase in the dose of cortisone. The relative magnitude of this coefficient is characteristic throughout the experiments of each investigator, and the average coefficient for the cortisone experiments is given at the foot of the table.

Venning *et al.* made particular mention of the purity of their strain. The coefficient of variation in some of their experiments was as low as 8%. The average coefficient for the whole 354 mice reported in their paper is 35%, and if the control groups are excluded, this falls to 25%. The average coefficient of variation for the mice of Dorfman *et al.* (1946) is far greater and suggests a fair admixture of strains.

The coefficients of variation were also calculated from the data of Olson *et al.* (1944) and of Pabst *et al.* (1947), who used the glycogen assay method in the rat, and the average figures in their assay of cortisone are 29.57 and 23.25% respectively. Rats are more easily obtained in reliably pure strains than mice, and it is evident that the accuracy of bioassays carried out with pure strains of rats or mice are of the same order.

TABLE 8. COMPARATIVE STUDY OF THE COEFFICIENT OF VARIATION OF THE MOUSE GLYCOGEN METHOD OF ASSAY OF GLUCO-CORTICOID ACTIVITY

(Values calculated from published results of various authors)

Dose of cortisone in μg.	Name of author			
	Nissim	Dorfman *et al.* (1946)	Venning *et al.* (1946)	Eggleston *et al.* (1946)
0 (control)	178.5	101.1	71.0	54.0
10	—	—	23.85	—
20	120.0	75.0	14.5	—
40	116.0	53.75	23.3	—
50	—	—	21.63	—
80	84.5	41.7	—	—
average variation of cortisone	106.825	56.8	20.825	—

TABLE 9. COMPARISON OF VALUES OF COEFFICIENT OF VARIATION, SLOPE, AND INDEX OF PRECISION FOR CORTISONE IN BIOASSAYS CARRIED OUT BY DIFFERENT AUTHORS. THE TABLE ALSO SHOWS PROPORTIONALITY BETWEEN SQUARE ROOT OF MEAN SQUARE OF ERROR(S) AND COEFFICIENT OF VARIATION

	Name of author			
	Nissim	Dorfman *et al.* (1946)	Venning *et al.* (1946)	Eggleston *et al.* (1946)
Average of coefficient of variation for cortisone	106.825	56.8	20.825	—
Slope of line of regression of cortisone (b)	12.9	22.23	9.59	8.1
Index of precision (λ) or s/b	0.505	0.158	0.137	—
Square root of mean square of error(s), calculated from line 2 and 3 above	6.523	3.512	1.314	—
s/v ratio	0.06105	0.0618	0.0631	—

s/v ratio approximately constant.

The index of precision and the accuracy of bioassay methods. Table 9 presents a comparison of the coefficient of variation, the slope and the index of precision for cortisone bioassays by Dorfman *et al.* (1946) and Venning *et al.* (1946) as well as in the present investigation. The slope represents the combined sensitivity of method and strain. The index of precision is the square root of the mean square of error divided by the slope, i.e. s/b, named λ by Gaddum (1933). The square of the index of precision is inversely proportional to the amount of information per test object, so that a smaller λ means greater precision.

Dorfman (1950) compared the index of precision of assays by Eggleston *et al.*, Venning *et al.*, and Dorfman *et al.* Eggleston's method D gave the lowest index of precision, and while the indices of Venning *et al.* and Dorfman *et al.* with cortisone were very nearly the same, that of Venning *et al.* was slightly smaller. The precision of the present experiments (0.505) is inferior to all three.

When the square root of the mean square of error (s) is calculated for the three experiments from the slope (b) and the index of precision (s/b), it is seen that they are in strict proportionality with the coefficient of variation (v). The ratio (s/v) gives the values 0.061, 0.062, and 0.063 for the three sets of experiments—an approximately constant figure. In the foregoing analysis it had been concluded that the average coefficient of variation for the cortisone experiments of each of the three workers virtually depended on the purity of the strain of animals used. The proportionality now established between the square root of the mean square of error and the average coefficient of variation shows that the error factor used in the calculation of the index of precision depends in practice on the purity of the strain.

Erroneous conclusions arrived at by using the index of precision (λ) *to compare merit of bioassay methods on animals of same species.* Dorfman (1949, 1950) presented a comparative study of the carbohydrate methods of assay of adrenal cortical hormones based on the value of the index of precision and on the error range of potency ratio. The error range of potency ratio is equal to $s/b\sqrt{1/n_1+1/n_2}$ as a first approximation, and is proportional to the index of precision. Dorfman (1949) concluded that "the method of Venning *et al.* (1946) appears to have the higher accuracy as seen by the fact that a lower error range of potency ratio was found with fewer animals," i.e. because of a smaller λ. Table 9 shows, however, that the lower index of precision obtained for the experiments of Venning *et al.* is due entirely to the smaller mean square of error. The value of the slope, which is proportional to the combined sensitivity of method and strain, is only 9.59 in the method of Venning *et al.* compared with 22.23 in the method of Dorfman *et al.* The calculated greater accuracy of Venning's method is due entirely to the uniformity of the strain. Unless the lower slope obtained by the Montreal workers is attributed entirely to a lower sensitivity of the particular strain of mice, Dorfman's method, as judged from the slope of 22.23, is the more accurate. The confusion of the precision of experiments with the accuracy of methods has obviously led to erroneous conclusions.

The fallacy resulting from such an assumption is perhaps best illustrated by exchanging the variation of the strain of mice in the two methods. By combining Venning's mean square of error[1] (s^2) with Dorfman's slope (b) and vice versa, the indices of precision would have assumed the following values:

a. Venning's mean square of error and Dorfman's slope, 1.314/22.23 = 0.0591
b. Dorfman's mean square of error and Venning's slope, 3.512/9.59 = 0.366

The indices of precision, far from being comparable, would have shown the ratio, 0.366/0.0591 = 6.2.

By combining Eggleston's mean square of error with Dorfman's slope, and vice versa, even greater differences in λ would have resulted.

Mouse versus rat glycogen methods of assay. Analysis of results led Dorfman to regard the rat glycogen method as "the mcst precise method available for the bioassay of adrenal cortical material but not the most sensitive." Pabst *et al.* (1947) also found that "the rat gives more reliable results," and pointed out that whereas Dorfman *et al.* (1946) found the error, with groups of 10 mice ($P = 0.05$), to be −59 to +150%, the error in their assays with groups of 10 rats is within 20%. These analyses did not take into account, however, the varied results obtained by different authors with different colonies of mice. In fact, the average coefficient of variation obtained by Venning *et al.* with a pure strain of mice is about the same as that of Pabst or Olson with rats. When this observation is coupled with the established greater sensitivity of mice to cortical hormones it is evident that greater precision should be attained with a pure strain of mice than of rats. Indeed, the index of precision for the assay of cortisone by Venning *et al.* is 0.137 compared with 0.149 for the assay of the same substance by Pabst *et al.* The index of precision for the assay of 11-dehydrocorticosterone by Venning *et al.* (0.222) is, however, higher than that obtained by Pabst *et al.* (0.095) for the same substance. This was mainly responsible for giving a greater error range of potency ratio in the comparison of 11-dehydrocorticosterone to cortisone by the method of Venning *et al.* (−17, +20) than that obtained by the method of Pabst *et al.* (−10, +12). The numbers of animals used by Venning *et al.* were, however, only 73 compared with 90 used by Pabst *et al.* The average of the coefficients of variation for all the results of Venning *et al.* (263 mice), excepting the control mice, is 25, while the average of this coefficient for the results of Pabst *et al.* with cortisone, adrenal extract and 11-dehydrocorticosterone

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[1] The mean square of error (s^2) is the error due to random sampling—in tables of analyses of variance—divided by the corresponding degrees of freedom.

(169 rats) is 34. Even for 11-dehydrocorticosterone itself the coefficient of variation for Venning's mice is 29.07, compared with a coefficient of variation for Pabst's rats of 30.82. Venning's strain of mice, therefore, can give results which are not only more accurate but also at a far smaller level of dosage. The first essential for securing a high level of precision with mice is to ascertain the purity of the strain.

SUMMARY

The mouse glycogen method of Venning *et al.* (1946) was used for the assay of gluco-corticoids, and factors influencing liver glycogen under the conditions of assay were studied. Marked variation in liver glycogen levels of adrenalectomized control and injected mice was noted, and this was paralleled by wide variation in normal mice.

The average coefficient of variation for the cortisone assay was 106.8% as compared with 56.8% for Dorfman *et al.* (1946) and 20.8% for Venning *et al.* (1946). The relative magnitude of this coefficient is characteristic throughout the experiments of each investigator. In the present experiments this was shown to be independent of the adrenalectomy or the chemical assay of glycogen, and is therefore an index of the purity of the strain of animals used.

Erroneous conclusions were arrived at by authors using the index of precision to compare the accuracies of assay methods without attention to differences in the variation coefficient of the different colonies of mice. Variation in strain should not be allowed to confound the issue when the accuracies of methods are compared.

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METHOD OF ASSAY OF A SODIUM-RETAINING FACTOR IN HUMAN URINE[1]

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THE retention of salt and water in a number of clinical conditions has sometimes been attributed to the presence of an excess of a desoxycorticosterone-like substance possibly of adrenal cortical origin. It has also been suggested that other conditions, among them certain rheumatic diseases, may be associated with a relative excess of "mineralocorticoid" (Selye 1951). For some time the validity of these suggestions could not be investigated because a sensitive method for the assay of this material was not available. Dorfman, Potts and Feil (1947) found that the urinary excretion of Na^{24}, during the first 6 hours after its subcutaneous injection in adrenalectomized rats, could be reduced by pretreatment with as little as 1 μg. microgram of desoxycorticosterone-acetate (DCA). A dose-response curve was not given and the effects were not always reproducible. However, the principles suggested by these investigators have been used as a basis for a biological assay procedure by several groups of investigators. None of the modifications, with the exception of that of the present study, have retained the use of Na^{24} as an index of mineral activity. Deming and Luetscher (1950), Spencer (1950) and Marcus, Romanoff and Pincus (1952) measured total urinary sodium by use of a flame photometer, Kagawa, Shipley and Meyer (1952) measured urinary sodium by a microchemical technique, and Simpson and Tait (1952) measured the urinary Na^{24}/K^{42} ratio.

Sodium-retaining activity, as determined by one of the above mentioned methods, has been detected in crude lipid extracts of urine from patients with congestive heart failure and nephrosis by Deming and Luetscher (1950) and Luetscher and Deming (1951), and in the urine of patients with toxemia of pregnancy by Chart, Shipley and Gordon (1951). Studies performed at the University Clinic of the Royal Victoria Hospital, with the method described in this report, have yielded similar results in congestive

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heart failure (Singer and Wener—in press), nephrosis (McCall and Singer—in press) and toxemia of pregnancy (to be published). Although the chemical nature of the active material in these urinary extracts is not known, it is possible that it is similar to a "mineralocorticoid" recently isolated from beef adrenal extract by Grundy, Simpson and Tait (1952) and from adrenal venous blood from a monkey and a dog by Simpson, Tait and Bush (1952). Recently two subtsances have been detected in pregnancy urine with Rf values corresponding with two substances in the amorphous fraction of adrenal cortical extracts (Bush, de Courcey and Gray, 1952).

The present communication includes the description of a method for the assay of a sodium-retaining factor in human urine as well as some studies on the properties of the active material.

METHODS

Assay Procedure

Hooded male rats weighing 160 to 170 gm. at adrenalectomy were used in all experiments. The animals were maintained on a standard diet of Purina fox chow and were adapted to laboratory conditions for one day prior to operation. Room temperature was maintained as close to 80° as possible. The animals were bilaterally adrenalectomized by the dorsal route under light ether anaesthesia and were placed on saline drinking fluid for the first post-operative day and on distilled water for the second. Assays were performed 2 days after adrenalectomy. Rats which had lost 10 grams or more following adrenalectomy, or gained more than a few grams, were discarded. Each group of test animals consisted of 6 to 9 rats. A similar group of control animals was required for each day of testing. The test and control animals used on one day were equally distributed throughout all the cages in order to avoid variations due to the time of handling.

Food and water were removed one hour prior to the injection of the animals. The animals were attached to a board and given subcutaneous injections of the test substance or the solvent vehicle alone (0.1 ml. of absolute alcohol) and 1.0 ml. of an aqueous solution containing 3.5 mg. of sodium chloride with tracer quantities of Na^{24}. The animals were returned to their cages for the urine collection period after ligation of the urethra with heavy thread. Five hours after the administration of the radioactive sodium, the animals were killed by the intraperitoneal injection of Nembutal. The body wall was opened and the bladder exposed. A fine needle and syringe were used to remove the urine directly from the bladder. Bladder and syringe rinses were added to the urine. Aliquots (1.5 ml.) of the urine plus washes were plated onto cupped metal planchets, dried under an infrared lamp and counted in a Geiger counter (Berkeley Scaler Model 1000-B with Tracerlab G-M tube with mica end-window less than 2 mg./cm.^{2}). The mean sodium-24 excretion of the control animals was considered as 100% excretion and that of the test animals was expressed as a percentage of the mean control value. Although the urine assays were all performed in the above manner, studies on individual steroids included the injection of test substances in 0.25 ml. of corn oil or 0.25 ml. of 25% alcohol as well. When the steroids were injected in these two materials, they were given 1 hour before the administration of the radioactive sodium and the urine was collected for a period of 6 hours.

Preparation of Urinary Extracts

Urine was collected without preservative, refrigerated and extracted, generally, within two days. It was acidified to pH 1.5 and extracted three times with redistilled chloroform. The chloroform was then distilled from the residue *in vacuo* at low temperature (water bath temperature 40° C.) and the remaining dry residue dissolved in a small quantity of absolute alcohol and sealed in a glass ampule under light vacuum. The ampules were refrigerated until used. Extracts prepared in this manner are referred to as crude lipid extracts. When desoxycorticosterone was added to normal human urine, which was then extracted as described, recoveries of 85% and 100% were obtained. Neutral lipid extracts were studied on several occasions. These were prepared by washing the crude lipid extract, dissolved in chloroform, with small volumes of 0.1 N NaOH. This was repeated five times. After each washing, the sodium hydroxide was extracted once with an equal volume of chloroform which was then added to the rest of the chloroform extract. The alkali-washed chloroform extract was washed several times with distilled water until the washings were neutral to pH paper. Here again, each washing was back-extracted with chloroform. The neutral lipid extract prepared in this manner was stored in the same way as described for the crude lipid extract. Unless otherwise stated, an amount of extract equivalent to a 20-minute sample of urine was injected into each animal.

RESULTS

Effect of DCA

A sodium-retaining effect was obtained with DCA at all dosage levels studied. The dose-response curves obtained with DCA injected in absolute alcohol (0.1 ml.), 25% alcohol (0.25 ml.) and corn oil (0.25 ml.) are presented in Figure 1. A dosage range of 1 to 20 μg./animal has been stuided. Each point represents the mean value obtained from one to three experiments. The mean Na^{24} excretion of the test animals, expressed as a percentage of that of the control animals, has been plotted against the dosage per rat. Regardless of the differences in solvents and timing, similar curves have been obtained in each instance. When the ratio of the urinary Na^{24} of test to control animals was plotted against the log of the dose, a straight line was obtained for each curve. Significant differences were not observed in the regression coefficients (Tippett, 1941) or the means (Snedecor, 1948) of these lines. The regression line obtained for all studies with DCA is presented in Figure 2. This line is derived from a total of 127 test animals. Significant sodium-retaining effects were observed at the 2 μg. dosage level (P is <0.01) or higher. The free steroid, desoxycorticosterone, has been studied at the 2 and 20 μg. dosage levels and the effect was similar to that observed with the acetate esters at the same dosage levels.

Effect of Cortisone

The effect of cortisone in dosages ranging from 1 to 52.5 μg. per rat is presented in Figure 3. Similar conditions and solvents were used as with DCA.

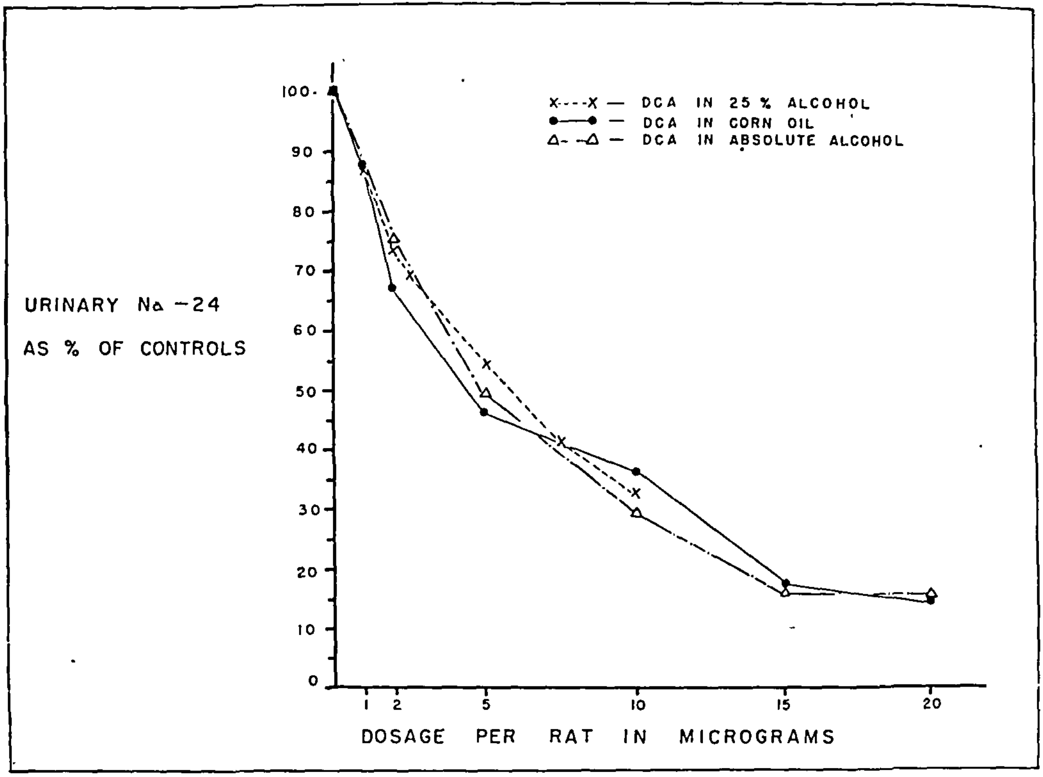

FIG. 1. Effect of DCA on urinary radiosodium excretion in rats. Curve for DCA in absolute alcohol obtained with 5 hour assay. Other curves obtained with 6 hour assay. Na^{24} excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention.

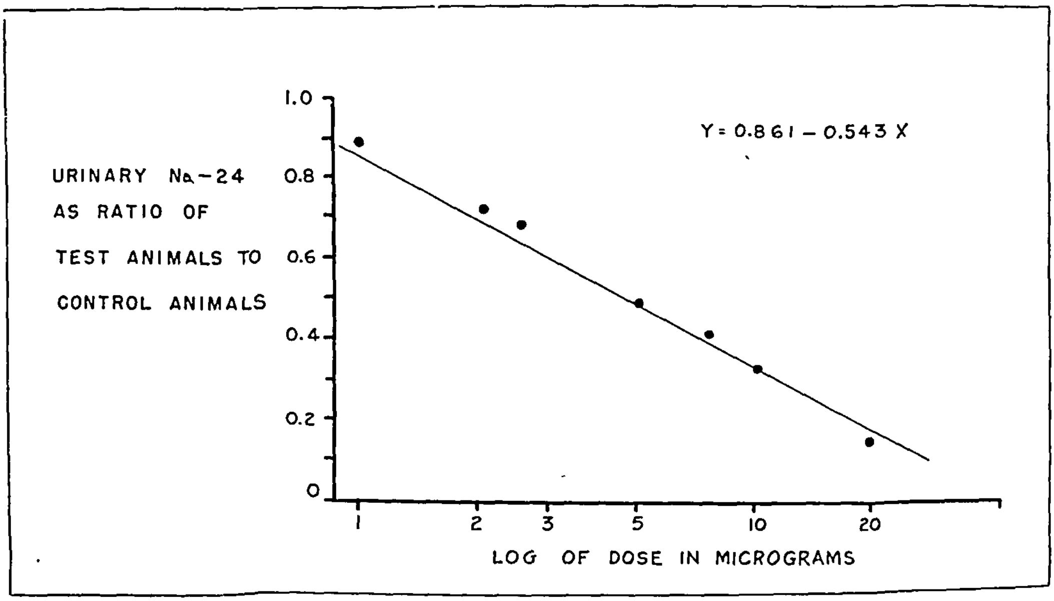

FIG. 2. Effect of DCA on urinary excretion of radiosodium in rats, log-dose-response relation.

Each point in the figure repersents the mean of a single group of 6–9 animals. This has been done because repeat assays at any one dosage of this material did not always give a similar effect. For the most part, animals treated with cortisone excreted more of the injected radioactive sodium than did the control animals, i.e. cortisone had a sodium-excreting effect. Lines have been drawn joining those dosages studied on the same day. Although these results do not give evidence of a clear-cut dose-response relation, they do suggest that the sodium-excreting effect increases with increasing dosage. Sodium-excreting effects were obtained throughout the entire range studied and in both the 6-hour assay (cortisone injected in corn oil and in 25% alcohol) and the 5-hour assay (cortisone injected in absolute alcohol). A sodium-retaining effect was observed on only two occasions and even then was not significant since the test animals excreted over 90% as much Na^{24} as the control animals.

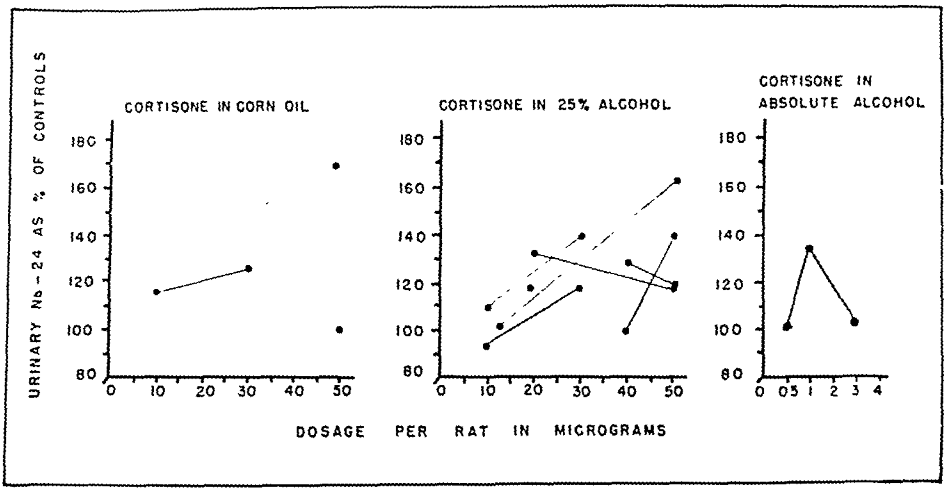

FIG. 3. Effect of cortisone on urinary excretion of radiosodium in rats. Na^{24} excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention. Experiments performed on one day are joined by lines. Each point represents the mean value for one group of rats. Studies with cortisone in absolute alcohol were done with the 5 hour assay, the others were done with the 6 hour assay.

Effect of Storage on the Activity of Urinary Extracts

Since the assays with radioactive material could not always be carried out immediately after the collection and extraction of the human urine, storage of the extracts for periods of several weeks was sometimes necessary. It was observed that a number of the extracts, which had been stored in the dry state at room temperature, lost a great deal of their sodium-retaining effect in 2 to 4 weeks. The change in activity of 4 extracts

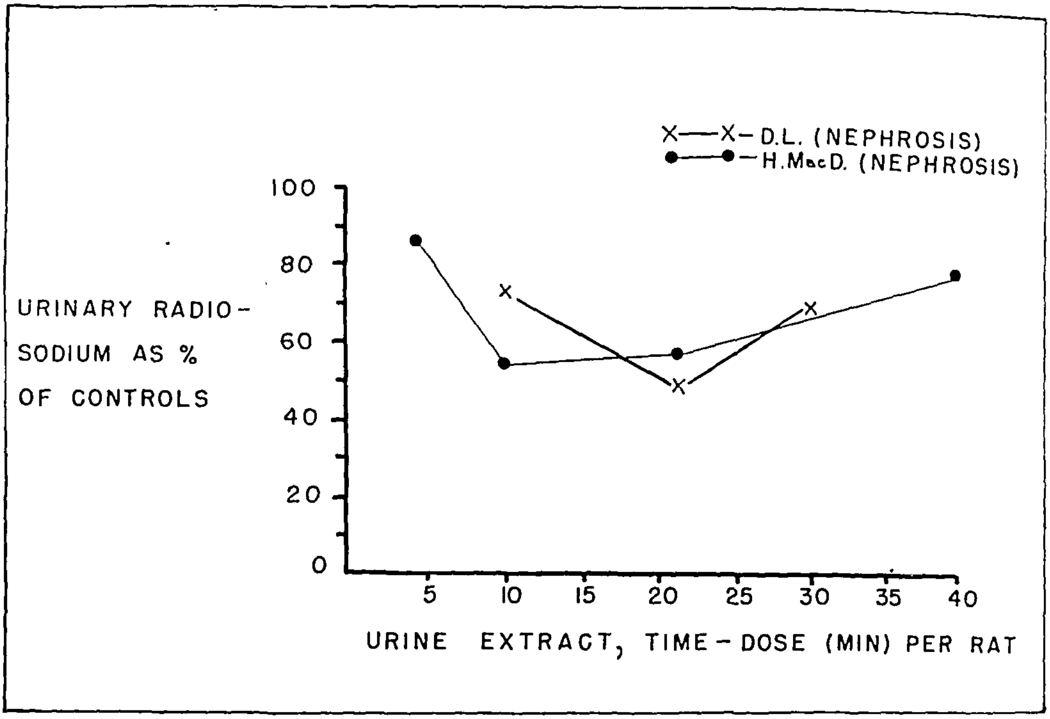

FIG. 4. Changes in activity of urinary extracts with time: effect of different methods of storage. Radiosodium excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention.

stored in this manner (referred to as "dry" storage) for several weeks is presented in Figure 4. The mean urinary radiosodium of the test animals, as a percentage of that of the control animals, has been plotted against the age of the extract in days. Losses in activity were observed in 3 of the 4 extracts. When the extracts were sealed in ampules in small amounts of absolute alcohol and refrigerated until assayed, losses during storage were negligible. Studies with three such extracts, stored for various periods of time, are presented in the same Figure. This method of storage is referred to as "wet" storage and was adopted for routine purposes.

Effect of Alkali and Water Washing on Activity of Urinary Extracts

Two urinary extracts obtained from patients with nephrosis were studied in the crude lipid form and in the neutral lipid form, i.e. after washing of the crude lipid fraction with dilute alkali and water. Results are presented in Table 1. These two studies suggest that the active material is relatively alkali-insoluble since recoveries of 114.3% and 81.7% were obtained in the washed chloroform fraction.

Effect of Variations in Time-dosage on Activity of Urinary Extracts

Two crude lipid extracts obtained from the urine or patients with nephrosis were studied at time-dosages ranging from 4.4 to 40 minutes per

TABLE 1. EFFECT OF ALKALI AND WATER WASHINGS ON SODIUM-RETAINING ACTIVITY OF CRUDE LIPID URINARY EXTRACTS[1]

Subject	Fraction	No. test rats	No. control rats	Urinary Na^{24} (% of controls)[2]	Approx. DCA equivs. (µg.)	
H.MacD.	Crude	5	7	57.1		4.4
H.MacD.	Neutral	6	8	50.0		4.9
					Recovery	114.3%
D.L.	Crude	8	10	49.7		4.9
D.L.	Neutral	6	6	58.5		4.0
					Recovery	81.7%

[1] Studies performed on urine of patients with nephrosis.

[2] Na^{24} excretion of control animals was considered as 100%. Values under 100% indicate sodium retention. DCA equivalents taken from curve obtained with DCA in absolute alcohol.

rat (Fig. 5). Results indicated that the sodium retaining effect was inhibited at the higher dosage levels. In one case (H.MacD.) studied at the 4.4, 10, 20 and 40 minute dosage, a maximum effect was reached at the 10 minute level. This degree of activity was maintained at the 20 minute dos-

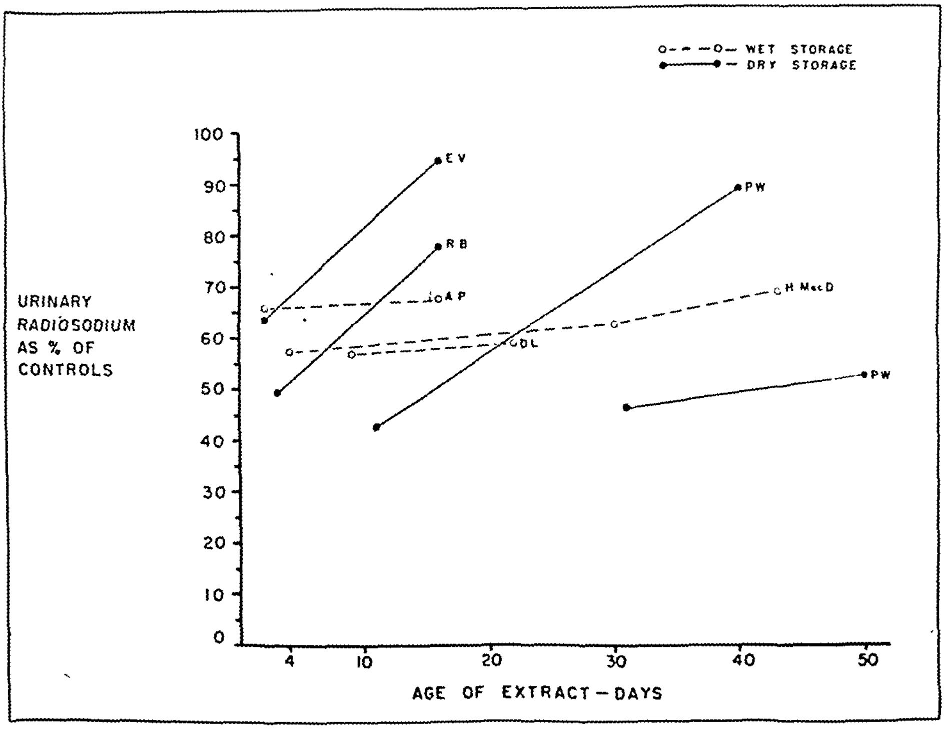

FIG. 5. Effect of different time-dosages of crude lipid extracts of urine on radiosodium excretion in rats. Na^{24} excretion of control animals was considered as 100% excretion. Values under 100% indicate sodium retention.

age but decreased again at the 40 minute level. The second extract (D.L.) which was studied at the 10, 20 and 30 minute dosage levels, showed a maximum sodium-retaining effect at the 20 minute level. The effects at the 10 and 30 minute dosages were similar.

Comparison of Method of Deming and Luetscher with Present Method

Dr. Deming and Dr. Luetscher kindly forwarded samples of two crude lipid extracts obtained from the urine of patients with nephrosis for comparison with the present assay. They were prepared by chloroform extraction of urine acidified to pH 1.5 immediately prior to extraction and stored in alcohol until assayed. Results obtained on these two extracts by Drs. Deming and Luetscher and by the present assay procedure are presented in Table 2. The extracts had marked sodium retaining effects in both assay procedures. Although some variation in the methods have been noted, results indicate that the order of sensitivity is within the same range in both methods. One extract, No. 4401, was more active in the assay described in this communication than it was in the laboratory of Deming and Luetscher (i.e. 10 μg. of DCA equivalents as compared with 6.2 μg.). Extract No. 2216 had less activity in the present assay than was observed by Deming and Luetscher (i.e., 4.3 μg. of DCA equivalents as compared with 7.3 μg.) but, since this extract was rather old when studied by the present method, this was not unexpected.

TABLE 2. SODIUM-RETAINING ACTIVITY OF TWO URINARY EXTRACTS BY THE METHOD OF DEMING AND LUETSCHER AND THE PRESENT ASSAY

Subject	Age of extract	No. test rats	No. control rats	Urinary Na^{24} (% of controls)[1]	Approx. DCA equivs. (μg.)
		Results obtained by Deming and Luetscher[2]			
2216					7.3
4401					6.2
		Results obtained in present assay			
2216	13.5 mos.	13	10	56.1	4.4
4401	1.0 mos.	14	14	27.6	10.0

[1] Na^{24} excretion of control animals was considered as 100%. Values under 100% indicate sodium-retention. DCA equivalents were taken from the curve obtained with DCA in absolute alcohol.

[2] Personal communication Dr. Q. B. Deming, November 6th, 1951.

DISCUSSION

A method for the detection of sodium-retaining material, based on principles suggested by Dorfman *et al.* (1947), has been standardized as a method for the bio-assay of sodium-retaining substances in human urine. The assay procedure is similar to those recently developed by several groups of investigators, and has a similar range of sensitivity to those methods employing adult rats (Deming and Luetscher, 1950; Kagawa, Shipley and Meyer 1952; and Marcus, Romanoff and Pincus 1952) although it is slightly less sensitive than those methods employing mice (Spencer 1950) or young rats 30 to 40 gm. (Simpson and Tait 1952). The similarity of the present assay with that of Deming and Luetscher is further illustrated by the fact that two urinary extracts had similar sodium-retaining effects in their hands and in ours.

In order to obtain reproducible results with DCA in the present assay, constant environmental conditions, weight of animals, pre and postoperative treatment as well as a constant procedure during the assay period were found to be essential. An important feature of this assay is that results must always be expressed in relation to control animals studied on the same day, as these do vary somewhat from day to day regardless of efforts to obtain constant conditions. All of the recently developed methods for the assay of substances affecting mineral metabolism stress the necessity of maintaining rigid conditions and most of them express results in relation to control animals studied on the same day (Simpson and Tait 1952, Kagawa *et al.*, 1952) or in relation to values obtained in the same animals serving as controls (Spencer 1950, and adopted later by Luetscher and Deming 1951). It is apparent from the present study, as well as those of others, that the excretion of sodium in adrenalectomized animals is quite variable and that for best results a group of at least eight animals should be used for each unknown.

The fact that cortisone generally has a sodium excreting effect in the assay, compared with that of DCA, introduces some difficulty in the interpretation of negative results in assays of urinary extracts, since these extracts also contain cortisone-like material. This effect of cortisone may have some bearing on the fact that a decreasing sodium-retaining effect is observed with increasing time-dosages, although the effect of other substances has not been ruled out. Until more highly purified extracts possessing a more consistent dose-response relation can be prepared, absolute results in urinary extracts must be evaluated with caution.

With regard to the possible interfering effect of cortisone in the assay, it would appear that the use of the urinary Na^{24}/K^{42} ratio, as described by Simpson and Tait (1952), may be useful in studying this effect. In their method, all the adrenal steroids tend to reduce the ratio in varying degrees. Thus, if a urinary extract contained both cortisone-like and "mineralocorticoid" material, one might expect that the effects would not cancel each other out but would, in fact, be additive. This point has not, however, been investigated. The quantity of cortisone-like material present in an extract could then be determined by other means if one were interested in knowing whether a separate "mineralocorticoid" were present in the unknown. The difference in the effect of cortisone in the present assay and that of Simpson and Tait is probably related to the fact that these investigators study the change in the ratio of Na^{24} to K^{42}, whereas the present method is based on the Na^{24} excretion alone. It is quite possible that cortisone may increase the urinary excretion of both of these substances but that the effect on potassium may be slightly greater, resulting in the observed fall in the ratio. The short urine collection period (2 hours) plus the low salt load administered to the animals by these investigators may also have some bearing on the effects of cortisone in their assay.

Since the chemical nature of the sodium-retaining factor in urine is not known, the reason for the apparent destruction of this material following "dry" storage cannot, at the moment, be ascertained. In this connection, it may be of interest that certain common molds can be used for the oxygenation of desoxycorticosterone, 17-hydroxy-11-desoxycorticosterone and progesterone (Colingsworth, Brunner and Haines 1952, and Peterson and Murray 1952). If the sodium-retaining factor in urine is a desoxy compound of this type, it is possible that this kind of reaction may have been responsible for the lability of extracts which were stored in the "dry" state at room temperature. The fact that losses were considerably smaller after storage in absolute alcohol with refrigeration lends some support to the suggestion that the decrease in activity of urinary extracts may have been due to a microbiological agent.

SUMMARY

A method for the detection of small quantities of sodium-retaining material has been standardized and applied to the study of crude lipid extracts of human urine. The method is based on the excretion of a dose of Na^{24} by adrenalectomized animals in a 5 hour period.

DCA had a sodium-retaining effect in the dosage range of 1 to 20 μg. per animal. A linear relation was observed between the log of the dose and the response. Significant sodium retention was obtained at the 2 μg. dosage level and higher.

Cortisone had mild to marked sodium-excreting effects which were not always reproducible, in the range of 1 to 52.5 μg. A consistent dose-response relation was not observed.

Urinary extracts could be stored for periods of at least 6 weeks without serious loss in activity if they were dissolved in alcohol, sealed in ampules and refrigerated until assayed.

The sodium-retaining effect of two crude lipid extracts of urine were not significantly altered by dilute alkali and water washings.

Dosage-response curves of two crude lipid urinary extracts indicated the

presence of interfering substances at the 20 minute level in one case and the 30 minute level in the other.

Acknowledgments

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EFFECT OF STIMULATION OF THE REPRODUCTIVE ORGANS OF THE COW ON THE RELEASE OF AN OXYTOCIN-LIKE SUBSTANCE

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FOR many years it has been recognized that there is a close association between the reproductive system and the mammary gland, however the physiological connection between the two has not been well defined. Several reports in the literature suggest that the ejection or "let-down" of milk in the mammary gland can be caused by the stimulation of the reproductive organs. This is not entirely a neural connection since there can be an ejection of milk even when the efferent nerves to the mammary gland have been cut (Ingelbrecht, 1935; Ely and Petersen, 1941; Espe, 1947). Generally it is accepted that the principal cause of milk ejection in the intact animal is the action of oxytocin on the mammary gland.

The purpose of this study was to determine the effect of stimulation of the reproductive organs of the cow by artificial insemination techniques and natural mating on the release of oxytocin as measured by increased intramammary pressure.

REVIEW OF LITERATURE

Some evidence suggesting a relationship between stimulation of the reproductive organs and milk ejection is found in the work of Nuesch (1904). In a review of literature, he states that a publication in 1727 refers to a practice of the Hottentots of blowing air into the vagina to bring about milk ejection in the absence of the calf. Nuesch also states that the Skyths of India milked mares in a similar manner. He further states that the ejection of milk in the cow can be brought about by rinsing the vagina or uterus, blowing air into the vagina, or even the manual removal of afterbirth. Tgetgel (1926) reported that manual manipulation of the uterus and ovaries of a cow caused milk ejection as did the presence of another cow in estrus. It has also been noted in our work that palpation of the uterus caused milk to drop from the teats. Hammond (1936) made the observation that in lactating mares, milk often flows freely during mating.

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METHODS AND MATERIALS

The experimental animals were 17 non-pregnant cows which were in their first to eighth lactation and were producing 20–67 pounds of milk daily. The experiments were done 7–11 hours after milking to allow time for milk to accumulate in the udder. The intramammary pressure was measured by a water manometer connected to a teat cannula which was inserted into the teat or gland cistern. Readings were taken with the cows in the same position each time since the position of the legs in reference to the udder is an important factor in intramammary pressure. If there was an ejection of milk due to insertion of the test cannula, a period of 20–30 minutes was allowed to elapse so that the pressure would return to normal or near normal.

In a preliminary study, 8 cows were tested at various times during the estrous cycle. Trials were made on 9 additional cows on the following days of the cycle: 1, 2, 4, 12, 16, 18, and 20 with the day of estrus being considered as day 1. The intramammary pressure was noted at one-minute intervals during massage of the vulva, massage of the cervix and cervical os through the rectal wall and during the insertion of a 7–8 mm. glass inseminating tube into the cervix. Also the pressure was noted at intervals after natural mating on the day of estrus.

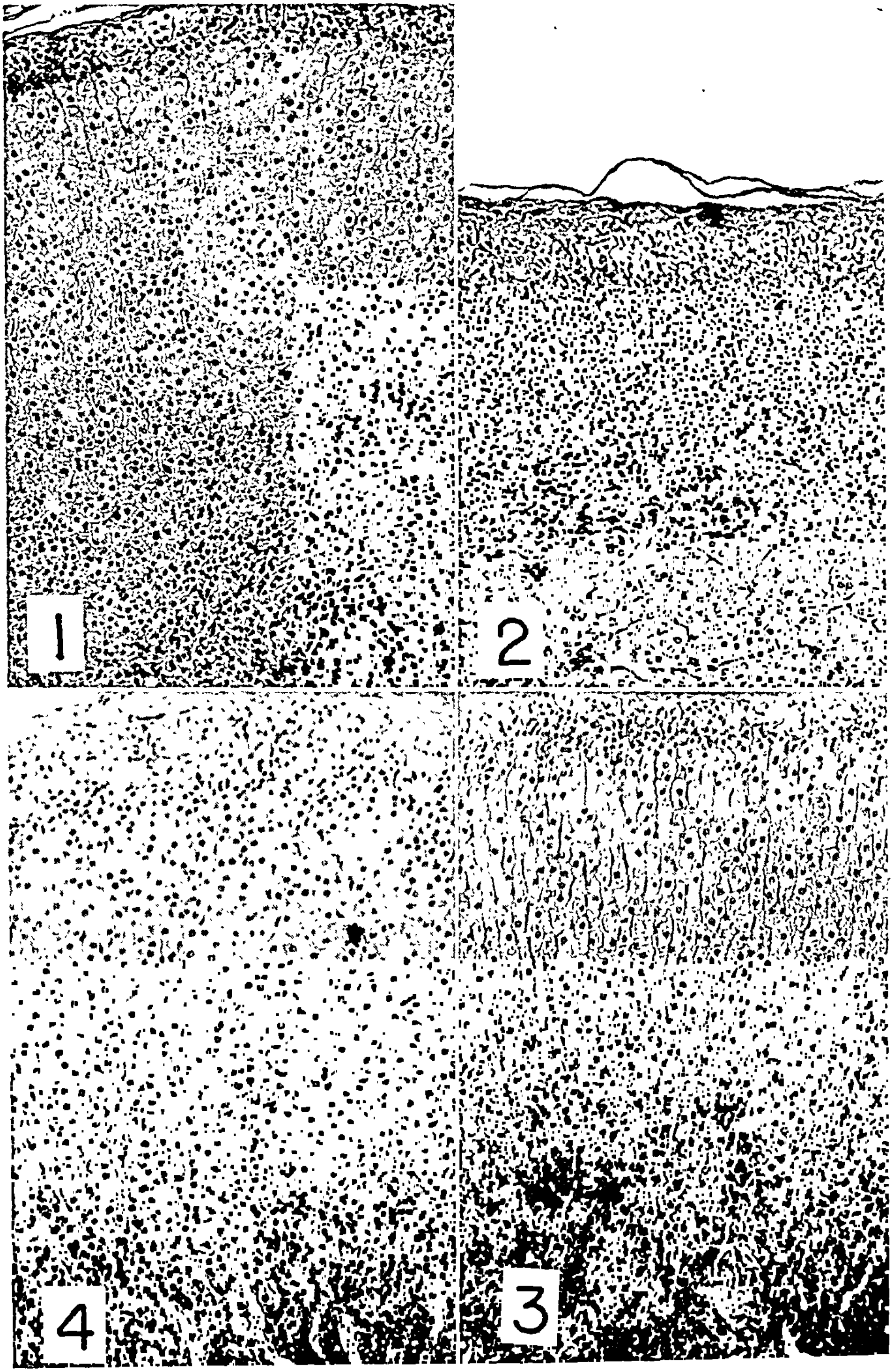
In order to prove that oxytocin or an oxytocin-like factor was present in the blood after stimulation, three pairs of blood samples were assayed with different excised cow uteri as test organs. The uterus was kept in a bath of Tyrode's solution at 39° C. and perfused with Tyrode's solution. To the perfusate was added samples of approximately 250 cc. of blood, taken before and after stimulation of the vulva, cervix, and uterus by palpation. The motility was determined by inserting into the excised uterus a balloon which was connected to a tambour. The contractions were recorded by a kymograph.

RESULTS

In 99 trials on 16 cows the artificial insemination techniques caused an increase in intramammary pressure in 88% of the trials. An increase of 1.5 cm. or more of water was considered a positive response. The trials which gave a positive response showed an average increase in pressure of 8.0 ± a standard error of the mean of 0.62 with a range of 1.5 to 31 cm. of water. Every cow gave a positive response in some or all of the trials. The results of the experiment on the 9 cows are shown in Table 1. The difference due to cows was highly significant. The differences between responses on different days of the cycle were not significant.

The increase in pressure following the different stimuli varied between cows and from time to time in the same cow. The response was caused by stimulation of the vulva, stimulation of the cervix by massage or stimulation of the cervix by the inseminating tube. In many trials the total response was due to a combination of two or more of the above stimuli. In 15 natural services 80% gave a positive response for an average increase in pressure of 4.5 cm.

Blood taken before stimulation of the vulva and cervix by massage had no effect on uterine motility when perfused into the excised uterus. But perfusion of the three blood samples taken after stimulation caused the



FIGS. 1–4

(cortisone, s.c.) no thymic tissue could be found at autopsy. The adrenal weights of Group 2 averaged 39.7 gm., which was not significantly different from the controls. The adrenals in Group 4 (cortisone, s.c.) weighed an average of only 15.2 mg., which is a highly significant difference from the adrenal weights of the controls. Cortisone had no effect on the weights of thyroid, ovary, uterus or hypophysis in either of the treated groups.

In the second series of male animals, Group 5 served as a weight control for Group 6 (cortisone, i.p.) and Group 7 served as a control for Group 8 (cortisone, s.c.). Again we found that Group 6 (cortisone, i.p.) gained an average of 33 gm. per rat per 40 days, which was not significantly different from the weight gain of the control group, whereas Group 8 (cortisone, s.c.) lost an average of 35 gm. per rat. The thymus and adrenal weights of Group 6 did not differ significantly from the controls, whereas there was no discernible thymic tissue in the animals of Group 8 (cortisone, s.c.) and the adrenals of this group were only 14 mg., compared to 33.8 mg. for the adrenal of the controls. Cortisone by either route of administration had no effect on the weights of the thyroid, testes, seminal vesicles, prostate or hypophysis.

Histologically, cortisone administered s.c. produced marked atrophy of the fascicular and reticular zones of the adrenal cortex while the glomerular zones remained relatively intact. This occurred in both male and female animals. Representative sections from the adrenals of these female animals are reproduced in Figures 1–4. The changes produced by cortisone s.c. are similar to those reported by Greep and Deane (1949), Yoffey and Baxter (1949), and Stebbins (1950). Cortisone i.p., however, produced no significant change in the histology of the adrenal cortex. We found no significant changes in the histology of the thyroids, ovaries or testes of either cortisone treated groups. We were unable to find that injection of the diluent produced any pathological changes in the endocrine organs of either sex.

EXPLANATION OF FIGS. 1–4

FIG. 1. Adrenal of female rat No. 68 (Group 3) receiving 0.15 ml. of Cortone® diluent subcutaneously, daily for 48 days. The three zones of the cortex are of normal width. (×100)

FIG. 2. Adrenal of female rat No. 58 (Group 4) receiving a total of 127.5 mg. of cortisone acetate subcutaneously over a period of 48 days. There is marked decrease in width of the cortex, due primarily to a reduction of the zona fasciculata. It is not possible to clearly define the margins of the zona fasciculata and zona reticularis. A portion of medulla shows at the bottom of the picture. (×100)

FIG. 3. Adrenal of female rat No. 82 (Group 2) receiving a total of 127.5 mg. of cortisone acetate intraperitoneally over a period of 48 days. Cortex of normal width with three distinct zones. (×100)

FIG. 4. Adrenal of male rat No. 20 (Group 6) receiving a total of 150 mg. cortisone acetate intraperitoneally over a period of 40 days. Cortex of normal width with 3 distinct zones (×100).

DISCUSSION

From the above data it was clear that cortisone administered intraperitoneally to the rat produced neither cessation of growth, thymic atrophy nor adrenal atrophy. There seemed to be several possibilities to explain this. First, the cortisone might not have been absorbed intraperitoneally. This did not seem likely since we were unable to find any unabsorbed material in the peritoneal cavity of the animals injected i.p., whereas despite the finding of a relatively large amount of unabsorbed material under the skin of the animals injected s.c., these animals showed marked effects. Second, the intraperitoneal cortisone might be inactivated in some fashion. This was possible, but did not seem likely because of the known effectiveness of cortisone orally and the failure of the liver to inactivate the adrenal hormones produced by the adrenal transplanted to the spleen (Bernstein, 1950) or cortisone pellets placed directly in the spleen (de Andino and Rivero-Fontan, 1951). Further, Mello (1949), showed increased liver glycogen deposition, increased survival and increased resistance to cold following intraperitoneal administration of whole adrenal cortical extract to adrenalectomized rats, and Speirs (1951) has reported eosinopenia in adrenalectomized mice following i.p. cortisone. There is little doubt, therefore, that cortisone is not inactivated intraperitoneally. Why does it not produce metabolic effects comparable to subcutaneously administered cortisone? The third possibility is that i.p. cortisone may be absorbed so rapidly that it does not maintain a blood level long enough or high enough for maximum metabolic effects, such as growth inhibition or adrenal atrophy. Some basis for this thesis is found in the observation of Wilkins, *et al.* (1952) on the increased effectiveness of intramuscular over oral cortisone therapy in supressing adrenal function in congenital adrenal hyperplasia in children. It was therefore of importance to determine the intensity and duration of the effect of a single dose of cortisone administered s.c. and i.p. in rats.

To test the effect of a single large dose of cortisone in the rat, a bioassay was devised utilizing the principle of the restoration of water diuresis in the adrenalectomized rat, as suggested by Eversole, Gaunt and Kendall (1942). The procedure was as follows: 15 female albino rats weighing 150–160 gm. were adrenalectomized, divided into three groups of 5 animals each and maintained on a stock diet with 1% saline as drinking water. The assay was begun 3 days postoperatively. On the control day of the assay, the animals received a hydrating dose equal to 2.5% of their body weight of distilled water by stomach tube. Three hours later they received a second dose of 5% of their body weight of distilled water by stomach tube. They were then placed in metabolism cages and the urine output recorded every 30 minutes for three hours. On the day following the control run, at the time of the initial hydration (three hours before the measurement of their diuretic response to a water load) one group of 5 animals received 3.75 mg. of cortisone s.c., a second group of 5 animals received 3.75 mg. cortisone i.p., and the third group of 5 animals received 0.15 cc. saline s.c. The animals were then given the same dose of water by body weight for the next four days and the urine output determined. No further cortisone was administered. All animals were maintained on 1% saline at night. By the fifth day of water hydration the adrenalectomized control animals had become quite ill, but both cortisone treated groups looked well.

On each successive day following the administration of cortisone or saline, the total urine output per animal per 90 minute period, corrected for the weight of the animal, was divided by the output during the control period to obtain a "Diuretic Ratio" as follows:

TABLE 2. THE EFFECT OF CORTISONE INTRAPERITONEALLY AND SUBCUTANEOUSLY ON THE DIURETIC RATIO* IN ADRENALECTOMIZED RATS SUBJECTED TO A DAILY WATER LOAD†

Hours after injection	Untreated controls	Cortisone s.c.	Cortisone i.p.
Control	1.00	1.00	1.00
3	.594	1.16	3.42
27	.565	1.25	2.00
51	.189	1.10	1.15
75	.087	2.18	1.13
99	0	1.06	.68

$$\text{* Diuretic Ratio} = \frac{\text{Mean 90 min urine output given period}}{\text{Mean 90 min urine output control period}}.$$

† See text for details.

$$\frac{\text{Mean 90 minute urine output given period}}{\text{Mean 90 minute urine output control period}} = \text{Diuretic Ratio.}$$

A Diuretic Ratio greater than 1 means a 90 minute diuresis greater than that during the control period, whereas a Diuretic Ratio of less than 1 means a diuresis less than during the control period.

The results of this study are shown in Table 2 and Figure 5. The untreated adrenalectomized animals put out less and less urine following repeated hydrations until at 99 hours the Diuretic Ratio fell to zero. The animals which received cortisone i.p. responded to a water load 3 hours after receiving cortisone with a large diuresis, so that the Diuretic Ratio at 3 hours after the injection rose to 3.42. Thereafter, the Diuretic Ratio fell slowly to about 0.7 at 99 hours. The animals which received cortisone s.c. maintained a constant Diuretic Ratio about 1 (i.e., the 90 minute urine output remained equal to the control day of the untreated adrenalectomized animals) until 75 hours when the Diuretic Ratio rose to 2.2, after which it fell at 99 hours to about 1.

These data would suggest that cortisone i.p. has an immediate effect on the restoration of water diuresis in the adrenalectomized rat. This effect is maximal on the day of injection, and gradually diminishes over the next

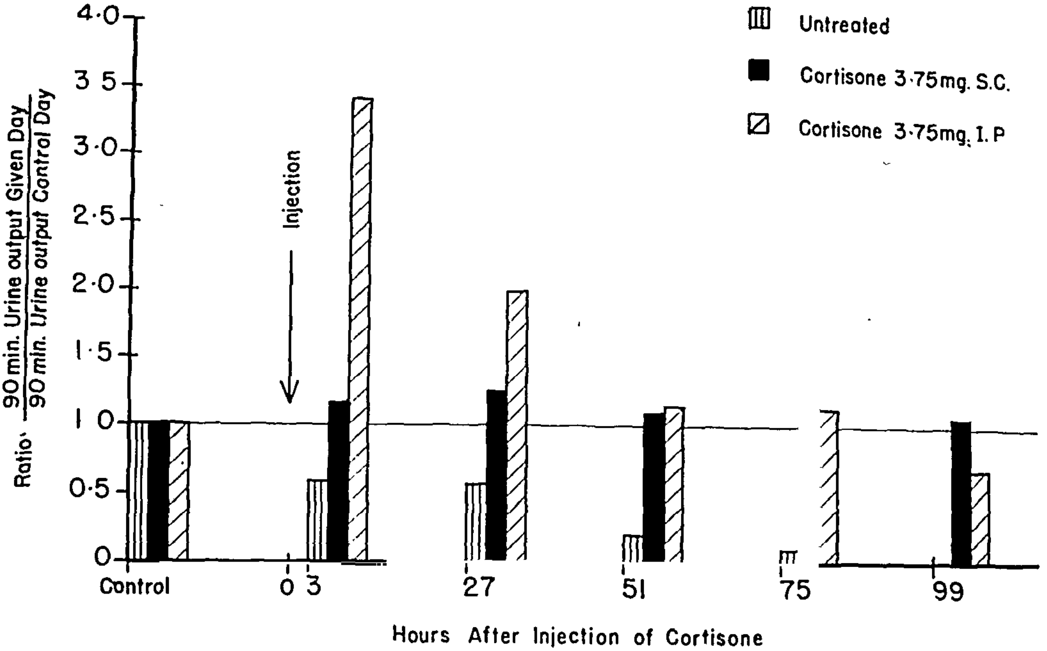


FIG. 5. The duration of effect of a single dose of cortisone, given intraperitoneally or subcutaneously, on the promotion of water diuresis in the adrenalectomized rat. (See text for details.)

four days. Cortisone s.c. had a slight effect for the first 3 days, but reached a maximum effect on day 4, after which the effect diminishes. Thus, there is no doubt that intraperitoneal cortisone has a definite and marked corticoid effect, as measured by this technique, and this effect is quicker, more intense, and of shorter duration than that produced by the same dose of cortisone given s.c. This is all the more remarkable since other corticoid effects such as growth inhibition, thymic atrophy and adrenal atrophy, as shown above, were not produced by intraperitoneally injected cortisone.

It would seem that in order to obtain maximum metabolic effects of crystalline cortisone acetate, a continuous high level must be maintained, such as might be expected from the continuous absorption of steroid from a subcutaneous depot. On the other hand, when intermittent high levels are induced, as with cortisone injected intraperitoneally, individual metabolic effects may be noted, such as the restoration of water diuresis in the adrenalectomized animal, but other effects, such as growth inhibition and adrenal atrophy may be absent.

SUMMARY

Cortisone acetate was administered intraperitoneally and subcutaneously to adult male and female rats in doses of 1.25 to 3.75 mg. daily for periods of 40 to 48 days. Subcutaneously administered cortisone acetate

produced the expected body weight loss, and atrophy of the thymus and adrenal cortex. The same dosage of cortisone acetate intraperitoneally did not cause weight loss, and produced no adrenal or thymic atrophy. The duration of effect of a single dose of cortisone was then studied by following the urine output of adrenalectomized rats subjected to a daily water load. It was found that intraperitoneal cortisone acetate had an immediate and rapid effect on the restoration of water diuresis in the water-loaded adrenalectomized rat, whereas subcutaneous cortisone acetate had a slower and more prolonged effect. The data suggest that certain effects of cortisone acetate, such as loss in body weight and atrophy of the thymus and adrenal cortex, may require a sustained corticoid level, as may be produced by subcutaneous administration of the drug. Other metabolic effects, such as the restoration of water diuresis in the adrenalectomized water-loaded rat, occur rapidly following intraperitoneal administration of cortisone acetate, but, perhaps because of the shorter duration of the effective corticoid level, there is no inhibition of growth or production of adrenocortical atrophy.

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THE EFFECT OF PROLONGED LOCAL TREATMENT WITH ADRENOCORTICAL AND SEX STEROIDS ON THE INTRADERMAL SPREADING ACTION OF HYALURONIDASE[1,2]

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THE inhibition of hyaluronidase activity as it affects the dermal spreading of an indicator substance by an acutely elevated blood level of either endogenously or exogenously supplied adrenocortical steroids (Opsahl, 1949 a,b,c; Winter and Flataker, 1950; Seifter, Baeder and Begany, 1949; Seifter *et al.*, 1950; Seifter, Baeder and Dervinis, 1949) has been confirmed (Hayes and Baker, 1951). Prolonged local application of whole adrenocortical extract, however, produced an accentuation in the activity of and an alteration in the pattern of spreading by the enzyme hyaluronidase (Hayes, Reed and Baker, 1950) when changes in area of spreading are measured as a function of time (Hayes and Reed, 1950). The change in enzyme activity was limited to the exact area of treatment and no systemic effect could be detected by this test. Employing pure crystalline steroids, Hayes and Bridgman (1951) reported that the prolonged local application of 11-dehydro-17-hydroxycorticosterone and desoxycorticosterone duplicated the results obtained with the prolonged local application of whole adrenocortical extract (Hayes, Reed and Baker, 1950). Progesterone, on the other hand, produced an accentuated spreading effect but with a different pattern of activity, quite unlike that obtained with adrenocortical extract or the adrenocortical steroids (Hayes and Bridgman, 1951). This report deals with an extension of these studies as related to specific pure crystalline steroids.

MATERIALS AND METHODS

The free alcoholic steroids selected for investigation were 17-hydroxycorticosterone (compound F),[4] corticosterone (compound B),[4] estradiol[5] and testosterone.[5] These sub-

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[2] The technical assistance of Mrs. A. D. Bridgman is gratefully acknowledged.

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[4] The supplies of free alcoholic compounds F and B and the hyaluronidase (Alidase) employed in this study were generously supplied by Dr. Irwin C. Winter, of G. D. Searle Company, Chicago, Illinois.

stances were dissolved in absolute ethyl alcohol at a concentration of 1 mg./cc.

Thirty-six litter mate male rats of the Long-Evans strain in the initial weight range of 200±50 gm. were used, kept in individual cages and fed a standard laboratory diet *ad libitum*. There were 6 animals in each of the 4 experimental groups; one group for each hormone. For 6 months 0.1 cc. of each of the steroid solutions was applied daily to the dorsal cervical region just caudal to the right ear of each of 6 animals. Twelve control animals were treated similarly but with only the solvent, absolute alcohol, in the same manner. The area of treatment in all animals was approximately 10 sq. cm. In all 5 groups (control plus experimental) the left side of each animal was left untreated. Body weights were determined and regrown hair on the neck was clipped at weekly intervals after hair growth patterns had been recorded (Butcher, 1934). The technic employed for study of the spreading action of hyaluronidase has been described in detail by Hayes and Reed (1950). The hair was clipped closely from the proposed injection sites on the dorsum of the neck and the animals were secured loosely for constancy of position. The animals were quieted by the subcutaneous injection of 0.3 cc. of a veterinary solution of sodium pentobarbital (6.5% solution) and were anesthetized lightly with ether only for the duration of the initial injection. The indicator employed was an isotonic solution of hemoblogin. The final injection quantity was 0.05 cc. containing 25 units of hyaluronidase.[4] The rate of injection was 0.01 cc./5 sec.

The manipulations of the injection procedure were controlled carefully and the *intradermal* injections were made in comparable sites on each side of the dorsal cervical midline, that is on the treated and untreated side of each animal.

Measurements were made of the length and width of the ellipsoidal area of spread. The first measurement, which was made at the termination of the injection, was taken as a baseline. Then, observations were made every minute for 15 minutes; every 5 minutes through 30 minutes; every 10 minutes through one hour and every 15 minutes through 2 hours. The increase in area for each time interval over that of the initial injection area was calculated, the arithmetic means were plotted using the ordinate as the increase in area of spread over the initial area in sq. mm. and the abscissa as the logarithm of the time in seconds.

OBSERVATIONS

Though no attempt at controlling food intake was made, it is significant that the control group mean weight gain over the period of study was 189 gm.; the compound F group mean weight gain was 184 gm.; the compound B group mean weight gain was 181 gm.; the testosterone group mean weight gain was 186 gm.; but, the estrogen group mean weight gain was only 18 gm. Of all the observations of spreading activity measured, 87% fell within a range of 4 sq. mm. of the plotted mean. There was no significant difference between observations made on the untreated and on the vehicle treated sides of the control animals. The results of the control tests are graphed as the X curve in all the accompanying illustrations. The effect of prolonged local treatment with testosterone and corticosterone

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[5] The estradiol and testosterone in free alcoholic form were supplied generously by Ciba Pharmaceutical Products of Summit, New Jersey.

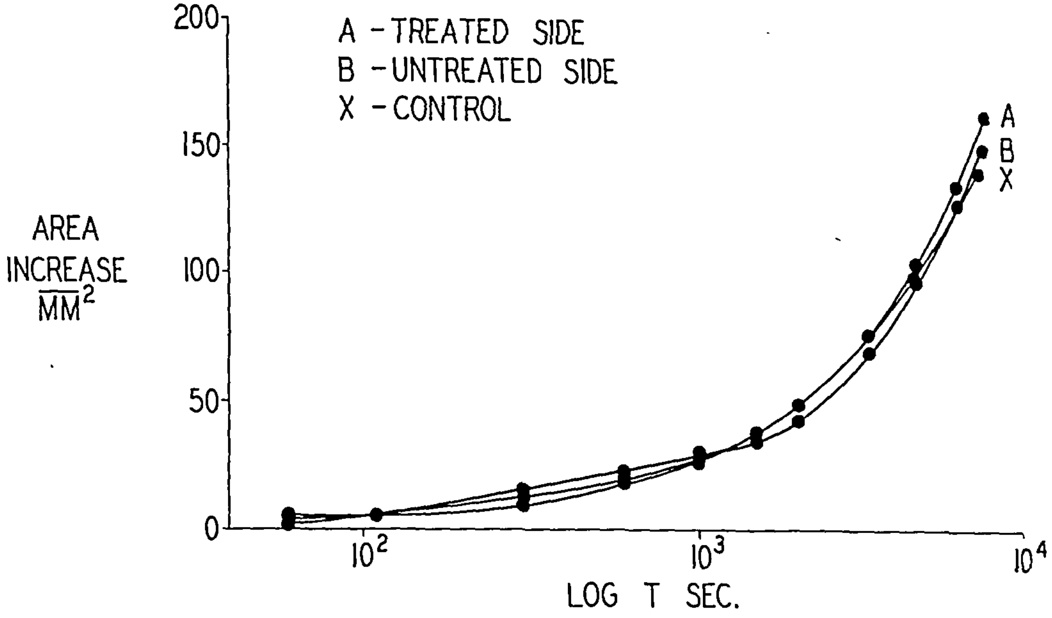

FIG. 1. Effects of the Chronic Local Administration of Testosterone. X is the control (see text). There is no significant difference between the treated and the untreated sides or either side and the controls.

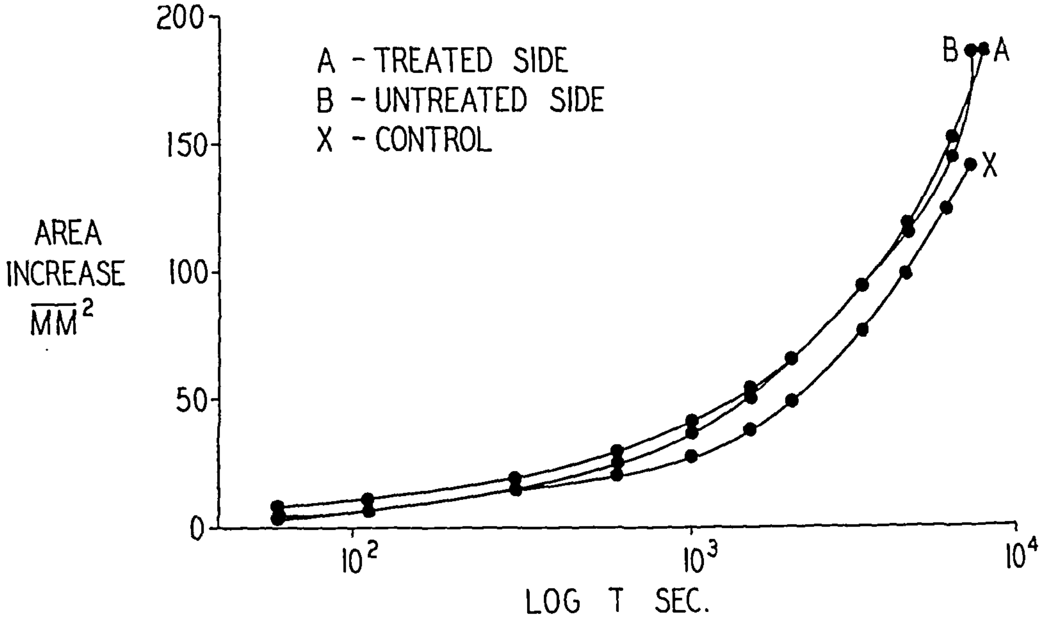

FIG. 2. Effects of the Chronic Local Administration of Corticosterone. X is the control (see text). There is no significant difference between the treated and the untreated sides or either side and the control.

on the usual pattern[6] of hyaluronidase intradermal spreading of a hemoglobin solution is well demonstrated in Figures 1 and 2. There is no significant alteration in either the pattern or extent of the spreading action. Figure 3 demonstrates the alteration in spreading caused by the prolonged local administration of estrogen. That there is a potent systemic effect is apparent since there appears to be no difference between the treated and untreated sides. In addition, the principal effect appears to be on the rapid phase of spreading and is comparable to the effect after similar treatment with progesterone (Hayes and Bridgman, 1951).

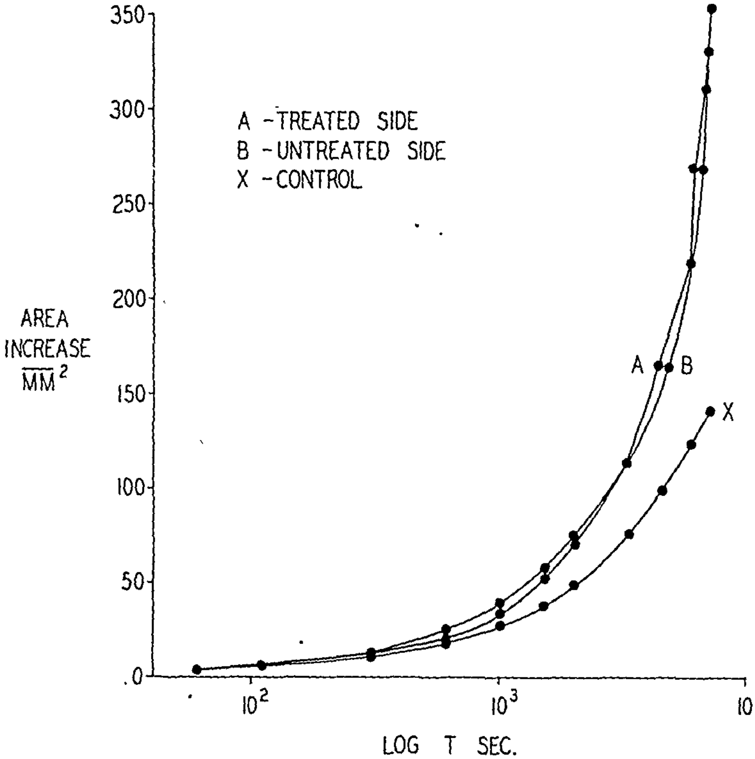

FIG. 3. Effects of the Chronic Local Administration of Estrogen. X is the control (see text). There is no alteration in the pattern of spreading but there is a very marked accentuation in rate of spreading in the rapid phase. There is no significant difference between treated and untreated sides, indicating a profound systemic effect.

In sharp contrast are the results obtained after the prolonged local application of 17-hydroxycorticosterone (Fig. 4). The slow phase of spreading is almost completely abolished and there is increased rate of spreading in the rapid phase.

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[6] All spreading activity is composed of two phases: an early slow phase followed by a late or rapid phase. Such a sequence is named pattern of spreading.

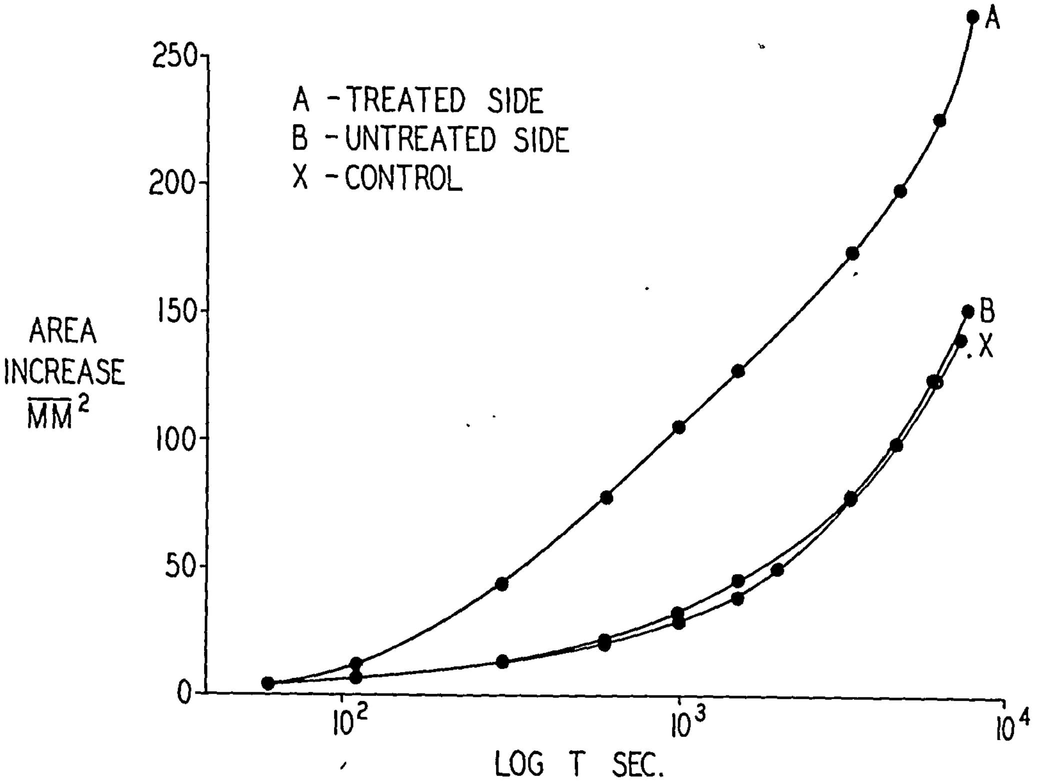

FIG. 4. Effects of the Chronic Local Administration of 17-hydroxycorticosterone. X is the control (see text). There is no significant difference between the control and the untreated side of the experimental animals. The treated side demonstrates an accentuation of spreading activity and a definite alteration in pattern of spreading, characterized principally by a change in the slow phase of spreading.

DISCUSSION

In this investigation prolonged local treatment with corticosterone or testosterone produced no alteration in the extent, rate or pattern of spreading. Prolonged estrogen local treatment resulted in a marked systemic response evidenced by growth inhibition and a striking alteration in rate of spreading; it is significant that there was no great alteration in the pattern of spreading. The use of 17-hydroxycorticosterone resulted in an alteration of rate and of pattern of spreading which has been reported to be characteristic with whole adrenocortical extract, whether applied locally for a prolonged period (Hayes, Reed and Baker, 1950) or given systemically in a chronic experiment (Hayes and Baker, 1951).

When the spread of an indicator without enzyme was studied in locally pretreated skin, no essential change in the manner or extent of spreading activity was observed (Hayes, Reed and Baker, 1950). This finding would exclude the thinness of the skin which exists under these conditions (Baker and Whitaker, 1948; Castor and Baker, 1950) as being the cause of the accelerated rate of spread. It is only reasonable to conclude, then, that such prolonged treatment with adrenocortical steroids alters the substrate, ground substance, in an unknown fashion rendering it more susceptible to enzymatic hydrolysis. It is of considerable significance that the ability to alter the slow phase of spreading appears to be a characteristic of only certain steroid hormones with an adrenocortical origin.

SUMMARY

Prolonged local application of corticosterone or testosterone does not influence the spreading action of hyaluronidase. Similar treatment with estrogen produces a profound systemic effect and accentuates hyaluronidase spreading, but only on the rapid phase. On the contrary, 17-hydroxycorticosterone produces an effect restricted to the area of treatment characteristic of adrenocortical steroids: accentuated rate of spreading and nearly complete abolition of the slow phase.

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THE EFFECT OF HYPOPHYSECTOMY ON THE REGENERATION OF THE LIVER AFTER PARTIAL HEPATECTOMY IN DOGS

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REGENERATION of the remaining portion of the liver after partial hepatectomy in the dog is rapid and complete within five to eight weeks (Mann, 1927; Fishback, 1929; Bollman and Mann, 1936; Grindlay and Bollman, 1952). Hypophysectomy was found to have little effect on regeneration of the liver in the albino rat if such factors as body growth and intake of food were considered. Franseen and co-workers (1938) and Higgins and Anderson (1931) and Higgins and Ingle (1939) came to the same conclusion, namely, that the retarded restoration of the remaining portion of the liver after the removal of the hypophysial body was largely if not entirely due to reduction in the amount of food consumed by the hypophysectomized rat. Canzanelli and associates (1949), in a more recent study, found great diminution in regeneration and content of nucleic acid of the liver after partial hepatectomy and hypophysectomy.

Because data on the effect of hypophysectomy on regeneration of liver in dogs were not found in the literature and because hypophysectomy affects very little the amount of food consumed by the dog, it was deemed desirable to study regeneration of the liver in dogs after hypophysectomy.

METHODS

Six adult dogs, apparently healthy and weighing from 7 to 12 kg., were used.

The pituitary body was removed by the transbuccal approach according to a modification of McLean's procedure (McLean, 1928; Essex and Astarabadi, 1952). The operation was considered successful when tissue characteristic of adenohypophysis (pars distalis) was not seen after thorough examination of a series of sections taken from the tissue remnant in the sella turcica at the end of the experiment. According to the criteria used only 3 dogs of the series of 6 were found to have had complete hypophysectomies.

Partial hepatectomy was carried out according to the method published elsewhere (Grindlay and Bollman, 1952). By this method about 70% of the liver was removed. Partial hepatectomy was done ten to twelve days after removal of the hypophysis cerebri.

The resected portion of the liver was weighed after the blood had drained out.

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Dextrose (50 gm. in 100 cc. of physiologic saline solution) was administered intravenously immediately before and 2 to 4 hours after partial hepatectomy. This was repeated twice daily until the dog resumed eating. It was found necessary to do this in order to prevent fatal hypoglycemia in the successfully hypophysectomized dogs.

The experiment was terminated by anesthetizing the dog, removing the remaining portion of the liver and weighing it in the same way as the portion removed at operation.

The amount of restoration was estimated by subtracting the calculated weight of the original remnant (which is 30% of the calculated weight of the whole liver or $\frac{3}{7}$ of the portion removed at operation) from the weight of the portion of the liver which remained at necropsy. To express the amount of restoration in per cent, the result of the subtraction was divided by the weight of the original remnant and multiplied by 100.

RESULTS

A significant difference was not found between the restored livers of the nonhypophysectomized dogs and those of the dogs which had a part or all of their adenohypophyses removed (Table 1).

TABLE 1. HEPATIC RESTORATION AFTER HYPOPHYSECTOMY AND PARTIAL HEPATECTOMY

Number and sex	Body weight		Liver weight, gm.		Per cent restoration	Days after hepatectomy	Conditions of adenohypophysis*
	Initial	Final	Removed	Final			
1 F	9.5	8.5	178	145	90	36	Absent
2 M	7	7.5	148	130	106	36	Absent
3 M†	12	11.5	140	200	233	47	Absent
Mean	9.5	9.2	152	146	143	39	
4 M	10	8.5	227	240	150	47	Partially regenerated
5 M	10.5	11	227	290	202	50	Intact
6 M	12	12	157	250	243	50	Regenerated
Mean	10.8	10.5	203	260	198	49	

* As revealed by histologic study of a series of sections from the tissue "remnant."
† Castrated.

Grossly, the restored livers looked the same in the two groups of animals. They were thick with rounded edges, showing conspicuous reticulation and firm consistency. The color of the livers, however, in the successfully hypophysectomized dogs was bright red, resembling the color of arterial blood or blood which had been saturated with oxygen, in contrast to the dark-red livers of the control dogs.

Histologic preparations of livers of control and hypophysectomized animals did not show any differences. The sections were cut ten microns in thickness and stained with hematoxylin and eosin.

The pancreas was decidedly pink in the hypophysectomized dogs as compared to the creamy color of the normal pancreas. This pinkish hue of the pancreas was reported by Sweet and Allen (1913) in hypophysectomized dogs and by Mann and Drips (1915) in adrenalectomized dogs.

It has been found that hypophysectomized dogs do not tolerate well preoperative fasting for 24 to 48 hours. A number of animals under investigation died in hypoglycemic coma when fasted for 24 hours. Whether fasting *per se* or the combination of fasting and the removal of 70 per cent of the liver was responsible for the fatal hypoglycemia, remains undetermined. Values for blood sugar as low as 15 mg. per 100 cc. of blood have been found in hypophysectomized dogs fasted for 24 to 48 hours.

COMMENT

The data obtained from the successfully hypophysectomized dogs indicated that the remaining portion of the liver may be regenerated in about 40 days to more than 140% of the calculated control weight in response to the removal of 70% of its mass. In other words, the 30% of liver remaining after partial hepatectomy will grow and after 40 days will weigh as much as the original liver. These findings are in accord with the work done on rats, in which forced feeding of hypophysectomized rats resulted in restoring the weight of the liver to normal both with and without partial hepatectomy (Franseen and co-workers, 1938; Levin, 1944; McQueen-Williams and Thompson 1939–1940). Therefore the reduction in the weight of·the liver which follows hypophysectomy is secondary to the decreased amount of food that is consumed by the hypophysectomized rat and is not caused primarily by the absence of a "specific splanchnotrophic" hormone of the pituitary body. That such a specific interdependence does not exist between the pituitary body and the liver in the dog is definitely shown by the results of this study.

The hypoglycemia which caused the death of many of the hypophysectomized dogs at the beginning of the investigation deserves comment. In a normal dog, removal of 70% of the liver is usually followed by rapid and uneventful recovery (Grindlay and Bollman, 1952). On the other hand, a hypophysectomized dog after removal of 70% of the liver has been found to manifest severe symptoms of hypoglycemia, namely, profuse salivation, weakness, generalized muscular twitching, and finally, if sufficient glucose is not given, convulsions, coma and death. The striking similarity of the symptoms just described to those of dogs after complete hepatectomy is recognized. In this respect hypophysectomy can be considered to have a decidedly adverse effect on the concentration of glucose in the blood after partial hepatectomy. Fasting for as brief a period as 24 hours likewise may, in certain instances, reduce the concentration of glucose in the blood of the hypophysectomized animal to a level incompatible with life. It is not surprising that the hypoglycemia of the hypophysectomized dog has been found to be greatly aggravated by partial hepatectomy.

It is possible that in the absence of the hypophysis cerebri, the change of the glycogen in the liver to the glucose in the blood is held in abeyance or is abolished altogether or the amount of glycogen which is contained in the residual portion of the liver after partial hepatectomy may not be enough to maintain the normal level of glucose in the blood. It is highly probable that in the absence of the hypophysis, the glucose of the blood is chiefly derived from the carbohydrate of the diet rather than from hepatic stores of glycogen. Severe hypoglycemia was not seen after partial hepatectomy if the dog had resumed eating. If the animals did not eat the food, glucose had to be given parenterally, or a fatal outcome was inevitable.

An adequate explanation for the intriguing colors of both the liver and pancreas of the hypophysectomized dogs cannot be given at present, but it seems questionable that they are produced entirely by changes in the state of the blood vessels of these organs.

SUMMARY AND CONCLUSIONS

The effect of hypophysectomy on regeneration of the liver after partial hepatectomy was studied in dogs, and it has been found that the absence of the hypophysis cerebri does not seem to interfere with regeneration of the liver.

Severe and sometimes fatal hypoglycemia developed in a few of the completely hypophysectomized dogs after fasting. It was hastened and aggravated by partial removal of the liver.

Changes in the color of the liver and pancreas have been noticed in hypophysectomized dogs.

Acknowledgment

The technical assistance of Mr. W. A. Meeker is acknowledged with thanks.

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STUDIES ON THE RATES OF INVOLUTION AND RECONSTITUTION OF LYMPHATIC TISSUE[1]

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MARKED lymphatic tissue involution has been demonstrated under conditions of inanition, irradiation and chronic treatment with adrenocortical hormones. Starvation provides certain unique advantages for the investigation of adrenocortically mediated acute lymphatic tissue involution. It is now known that: (1) loss of lymphatic tissue weight during a period of inanition begins earlier and proceeds at a more rapid rate than that of other body tissues (Jolly, 1914) and, (2) the rate of release of nitrogen from lymphatic tissue in the starved animal is under adrenocortical hormone control (White and Dougherty, 1947).

Although there is an extensive amount of literature on the involution of lymphatic tissue produced by inanition, little is known concerning its reconstitution. A series of experiments was designed to compare the influences of fasting and refeeding on lymphatic tissue of intact and adrenalectomized mice.

MATERIALS AND METHODS

A total of 515 CBA mice of both sexes between 12 and 16 weeks of age was used in these experiments. Animals were kept at constant temperatures and segregated according to sex. All mice that were fed were given dog chow (Purina) and water *ad libitum.* Fasted mice were kept in individual containers with screen bottoms to prevent coprophagy.

The control group consisted of 150 intact-fed mice. The experimental animals were divided into fasted and refed groups. The fasted animals studied were: (1) Intact mice fasted; different groups were killed at 24, 48, 72 and 96 hours. (2) Intact mice fasted 48 hours, then adrenalectomized and fasting continued. Groups of these starved, adrenalectomized animals were killed at 6, 12, 24 and 48 hours after operation.

Refed animals included: (1) Intact mice fasted 96 hours and refed for a period of 16 days, during which time groups of animals were killed on successive days. (2) Intact mice fasted 96 hours, adrenalectomized and refed for a period of 8 days. Groups of animals were killed at refeeding intervals of 2 days.

All animals were weighed at the beginning of the experiment and at the time of kill.

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TABLE 1. MG. LYMPHATIC TISSUE PER 100 GMS. FINAL BODY WEIGHT OF INTACT AND ADRENALECTOMIZED-FASTED MICE

Hours of fast	Intact fasted	Fasted adrenect*	Intact fasted	Fasted adrenect*
	Total lymphatic organ sample		*Thymus*	
0	356 ±11†		172 ± 7.5	
24	329.6 ±22		166.9 ±10.1	
48	174.3 ±14.5		72.0 ± 9.6	
54	—	258.0 ±14.0	—	120.0 ±10.8
60	—	218.6 ±23.8	—	89.3 ±12.4
72	178.0 ±10	226.0 ±14.9	63.2 ±6.8	90.0 ± 8.8
96	134 ±12	184.0 ±12.6	31.2 ±2.3	59.2 ± 5.4
	Spleen		*Mesenteric node*	
0	330 ±14.6		128 ±10	
24	231.9 ±16.7		119.8 ±11.4	
48	160.0 ± 8.9		68.5 ± 3.6	
54	—	218.0 ± 9.9	—	102.0 ± 9.0
60	—	193.0 ± 7.2	—	95.8 ± 9.4
72	142.2 ±10.4	221.0 ±14.6	83.2 ±6.6	94.0 ± 6.0
96	112.7 ± 8.3	184.0 ±11.6	87.5 ±9.6	87.0 ± 6.2
	Inguinal nodes		*Axillary nodes*	
0	28.5 ± 2.8		27.5 ± 2.5	
24	21.3 ± 1.1		21.6 ± 1.3	
48	18.0 ± 2.1		15.8 ± 2.2	
54	—	18.8 ± 1.8	—	17.4 ± 1.4
60	—	17.7 ± 1.7	—	15.6 ± 1.4
72	15.4 ± 1.2	21.8 ± 2.3	15.2 ±1.3	19.9 ± 2.1
96	8.0 ± 0.6	18.5 ± 1.5	7.4 ±0.5	19.3 ± 1.5

* Adrenalectomized after 48 hours fast and continued to fast.

† 0, 24, and 48-hour weights for both intact and adrenalectomized groups.

The lymphatic organs examined were paired inguinal, axillary and cervical nodes, thymus, mesenteric node and spleen. These organs were dissected free of connective tissue and weighed by means of a torsion balance. Both the individual lymphatic organ weights and the weight of the pooled lymphatic organs were obtained for each animal. The weights of the aggregates of lymphatic organs do not include the spleen and are referred to as the total lymphatic organ sample. Nitrogen determinations, however, were performed on the pooled total lymphatic organ samples and included the spleens. Nitrogen content was determined by semi-micro Kjeldahl analysis and is expressed as mgs. of nitrogen/gm. of lymphatic tissue.

Bilateral adrenalectomy was performed under ether anesthesia by the usual dorsal approach. Tail blood was used for all leucocyte counts. Differential blood counts of 200 cells were done on films stained with May-Grunwald Giemsa. Lymphatic organs (spleens, mesenteric nodes, thymi) from intact-fed, intact-fasted, and fasted-adrenalectomized mice were fixed in Zenker's solution and stained with methyl-green pyronin for histological study. Mitotic indices[2] were calculated by counting the number of mitotic figures per thousand cells in areas of the same cellular density.

Organs weights of refed mice were expressed in terms of 100 gm. of body weight of control-intact-fed mice of the same age and sex as the experimental animals at time of kill. Organ weights of fasted animals were expressed in terms of 100 gm. of final body weight of the experimental animal.

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[2] The authors wish to thank Mr. Gottlieb Schneebeli for his expert histological preparations and his aid in performing the mitotic indices.

RESULTS

Effect of fasting on lymphatic tissue of intact mice. It may be seen from Table 1 and Figure 1 that the weights of the total lymphatic tissue sample decreased rapidly during starvation. After a 96-hour fast the total lymphatic tissue sample had lost 63% of its initial weight as compared to a 32% loss of body weight. The maximum decrement occurred between 24 and 48 hours whereas the body lost weight at a fairly constant rate during the entire starvation period (Fig. 1).

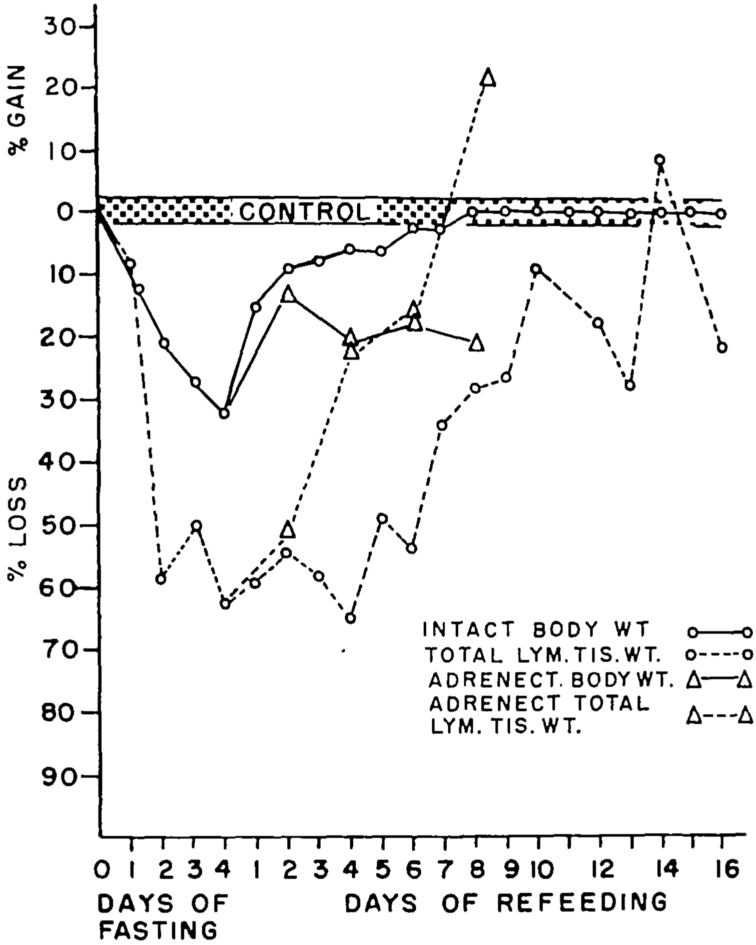

FIG. 1. Per cent weight changes of total lymphatic tissue weights and body weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days.

The responses of individual lymphatic organs to fasting are recorded in Table 1. Individual lymphatic organs differed in degree and rate of weight loss. The thymus proved to be the most sensitive lymphatic organ to inanition. It lost 66% of its initial weight by 48 hours and 83% by the end of the fasting period (Fig. 2). The spleen lost 66% of its initial weight by

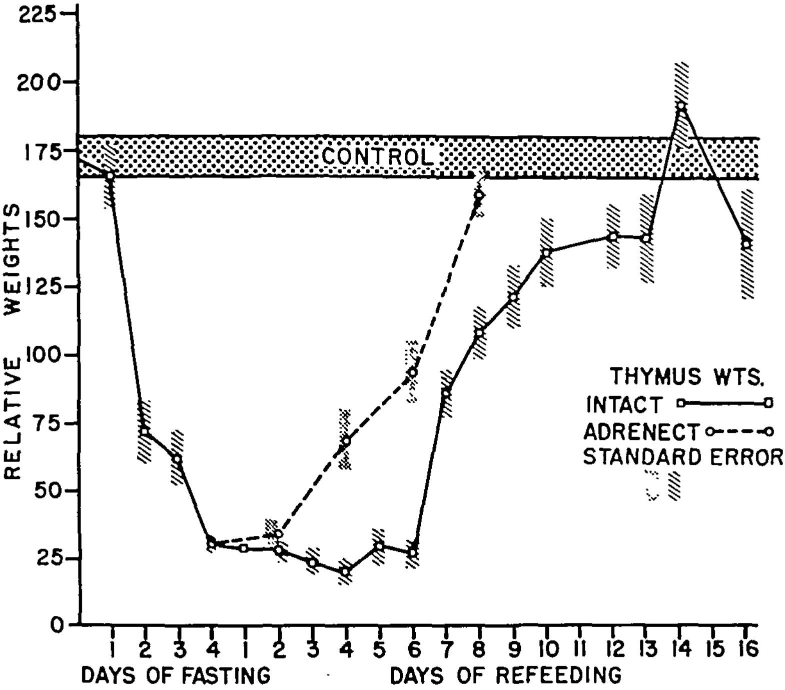

Fig. 2. Relative thymus weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

Table 2. Influence of fasting, adrenalectomy and refeeding on absolute numbers of leucocytes

Groups	Number of animals	Time of fasting or refeeding	W.B.C., thousands per cmm.	Lymphocytes, thousands per cmm.	Polymorphonuclears, thousands per cmm.	Eosinophils per cmm.
			Mean S.E.	Mean S.E.	Mean S.E.	Mean S.E.
Intact controls	150	—	12.6±0.3	8.6±0.3	3.9±0.1	117± 8.0
Intact fasted	10	24 hrs.	16.7±1.9	8.5±1.2	8.1±2.5	101± 37.0
	19	48 hrs.	13.9±0.9	5.4±1.0	8.6±0.8	34± 14.0
	10	72 hrs.	10.6±1.1	3.1±0.4	7.6±1.2	0±
	28	96 hrs.	11.3±1.0	5.2±0.7	6.1±0.6	28± 11
Adrenalectomized fasted*	14	54 hrs.	21.5±1.9	14.4±4.2	6.2±0.9	299± 69
	9	60 hrs.	23.5±2.5	19.6±2.1	4.0±0.8	15± 15
	9	72 hrs.	17.1±1.9	14.8±2.3	2.3±0.4	28± 19
	13	96 hrs.	24.1±7.1	17.1±3.6	7.0±1.2	43± 29
Intact refed†	10	1 day	13.0±1.4	5.8±1.3	7.1±1.1	50± 40
	10	2 days	11.4±0.8	4.4±0.7	6.9±0.9	30± 20
	10	4 days	14.8±1.4	10.5±1.1	4.3±0.5	20± 20
	12	8 days	20.6±1.1	14.3±1.3	6.1±1.3	150± 45
	10	12 days	12.4±1.3	8.0±0.8	4.3±1.2	80± 45
	5	16 days	20.1±2.7	11.9±1.0	4.9±0.9	200± 25
Adrenalectomized refed‡	13	2 days	15.8±2.5	11.9±0.6	3.8±0.5	79± 34
	9	4 days	16.0±2.5	10.5±1.4	4.9±0.6	104± 37
	8	6 days	20.3±2.2	11.6±1.1	8.3±1.2	463±119
	6	8 days	18.7±2.2	12.7±2.2	4.5±0.6	1285±438

* Adrenalectomized following a 48-hour fast and continued to fast.
† Previously fasted 96 hours.
‡ Adrenalectomized following a 96-hour fast.

96 hours (Fig. 3). The mesenteric node lost 48% of its weight by 48 hours but then gained so that by 96 hours only a 31% weight loss was evident (Fig. 4). Inguinal and axillary nodes began to lose weight during the second day and had lost 64% of their initial weight by the end of the fasting period (Fig. 5).

The ratio of nitrogen content per unit weight of lymphatic tissue remained constant during the entire fasting period (Fig. 6).

There was no significant change in the total leucocyte count during fasting. A polymorphonuclear leucocytosis was found at all time intervals. A lymphopenia and eosinopenia occurred by 48 hours of fast and persisted throughout the fasting period (Fig. 7, Table 2).

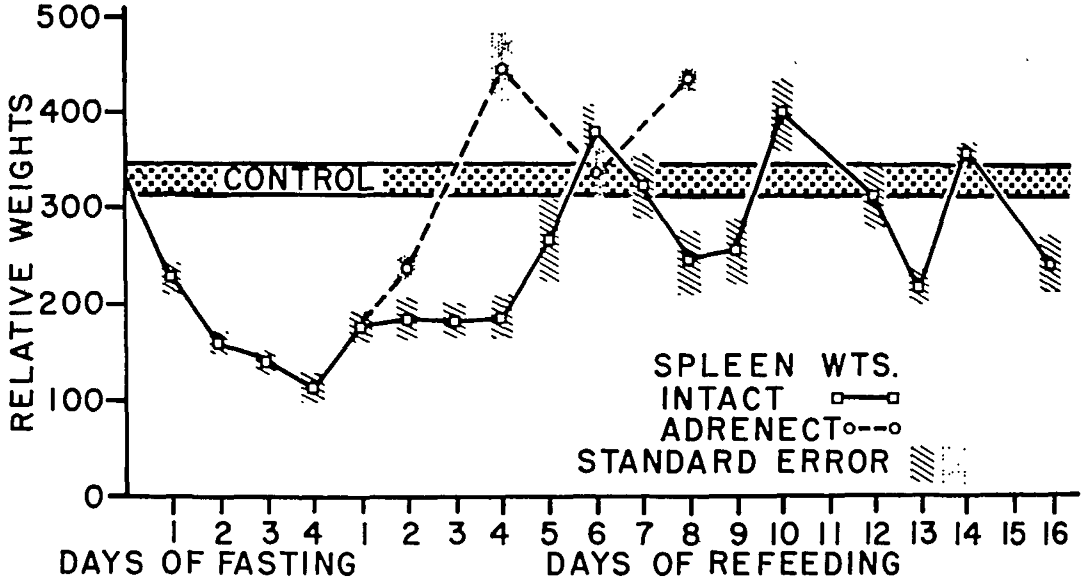

FIG. 3. Relative spleen weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

Effect of adrenalectomy on lymphatic tissue of fasted mice. As seen in Table 1 and Figure 8, adrenalectomy after 48 hours of fast arrested the involution of lymphatic tissue. In fact, 6 hours after operation an actual increase in lymphatic tissue weight was observed. Although 12 hours after operation (72 hours of fast) the total lymphatic tissue again began to lose weight, the loss of weight was still not as great as that of the intact animals by the end of the fasting period. Individual lymphatic organ weights were significanlty higher than those of the intact group at the end of the fasting period with the exception of the mesenteric node (Table 1).

A marked lymphocytosis (as compared to normal levels found in intact-

TABLE 3. NUMBER OF MITOTIC FIGURES PER 1000 CELLS (MITOTIC INDEX) IN MALPIGHIAN CORPUSCLES, THYMIC CORTICES AND GERMINAL CENTERS OF LYMPHATIC ORGANS IN FED, FASTED AND FASTED-ADRENALECTOMIZED MICE

Groups	Number of mice	Hours of fast	Mitotic index*		
			Spleen	Thymus	Mesenteric node
Intact fed	5	—	12.0	15.6	12.0
Intact fasted	5	48	5.2	0.4	8.0
	5	96	5.5	0.2	4.3
Adrenalectomized at 48 hours of 96-hour fast	5	54	3.0	3.0	10.6
	5	60	11.2	1.0	15.0
	5	72	16.0	3.6	15.4
	5	96	27.6	15.6	29.7

* Mean values for group (5000 cells/mouse).

fed animals) was observed 6 hours after adrenalectomy and was at a higher level at 12 hours (Fig. 9). At 24 hours (72 hours of fast) the circulating lymphocytes had decreased to the lymphocytotic level characteris-

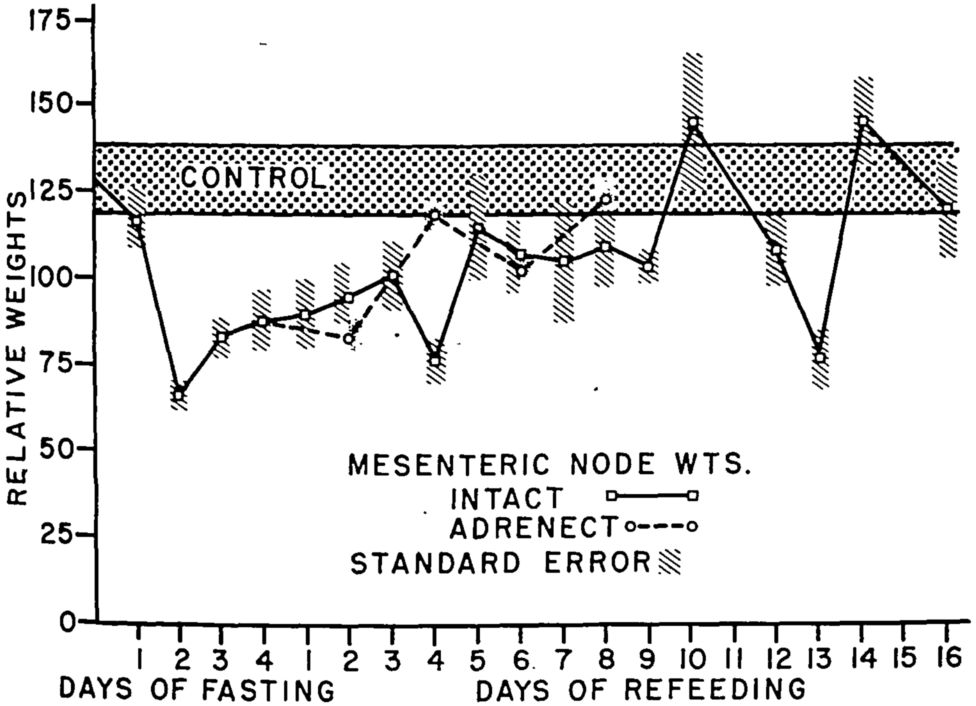

FIG. 4. Relative mesenteric node weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

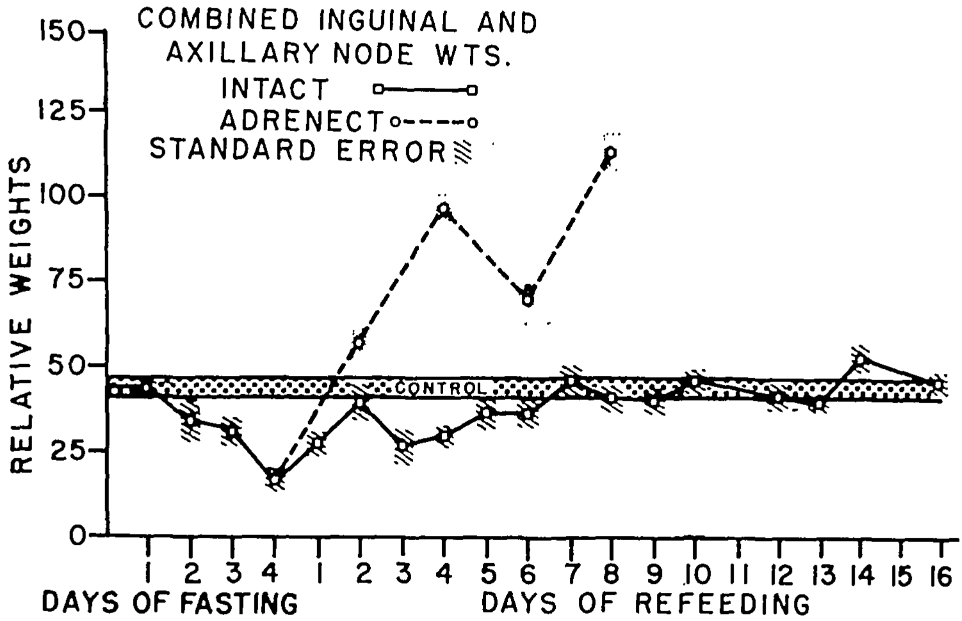

FIG. 5. Relative combined inguinal and axillary node weights of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days. Fasting weights expressed in mg. lymphatic tissue/100 gm. final body weight of experimental animal. Refed weights expressed in mg. lymphatic tissue/100 gm. body weight of intact-fed mice of the same age and sex as experimental mice.

tically seen in adrenalectomized-fed mice and remained at this level throughout the fasting period.

Effect of fasting and adrenalectomy on mitotic indices of lymphatic tissue.

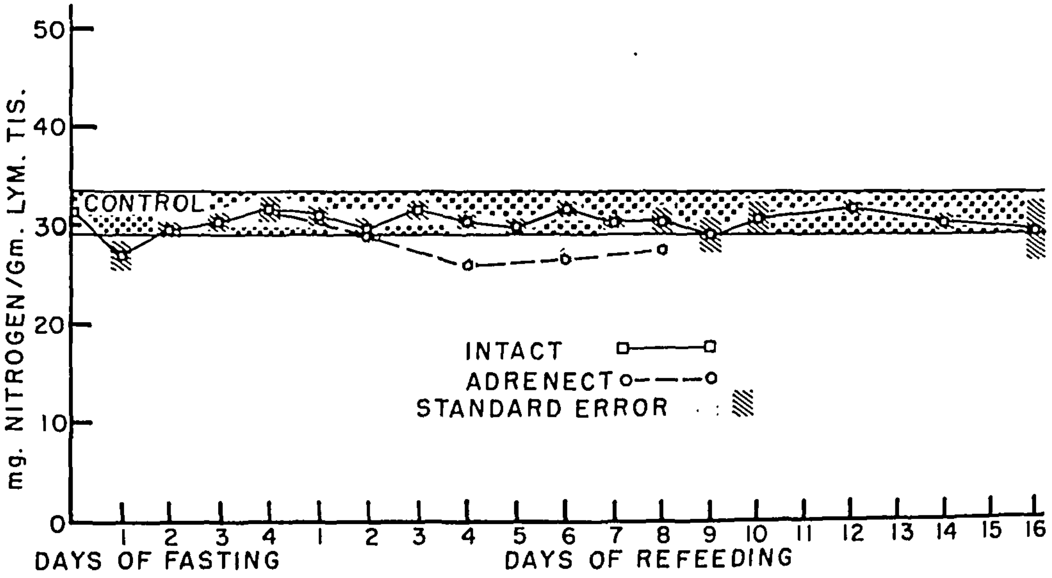

FIG. 6. Mg. nitrogen/gm. lymphatic tissue of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days.

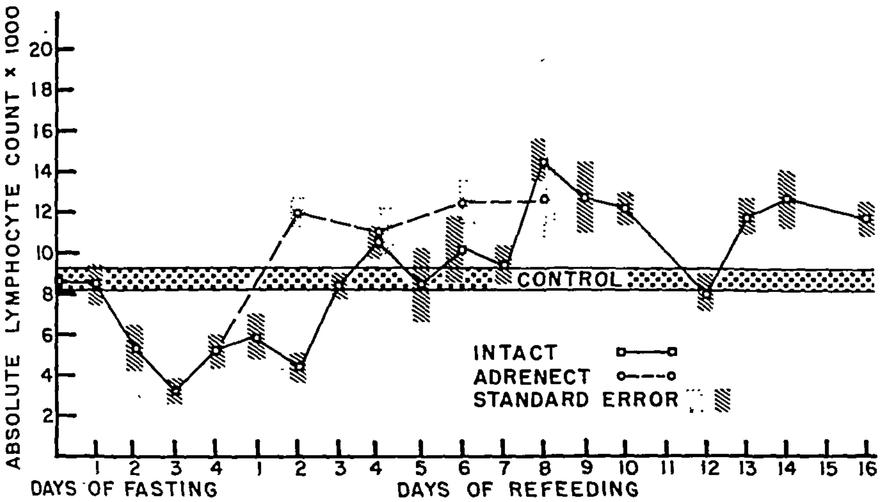

FIG. 7. Absolute lymphocyte counts of intact mice fasted 4 days and refed 16 days and of intact mice fasted 4 days, adrenalectomized and refed 8 days.

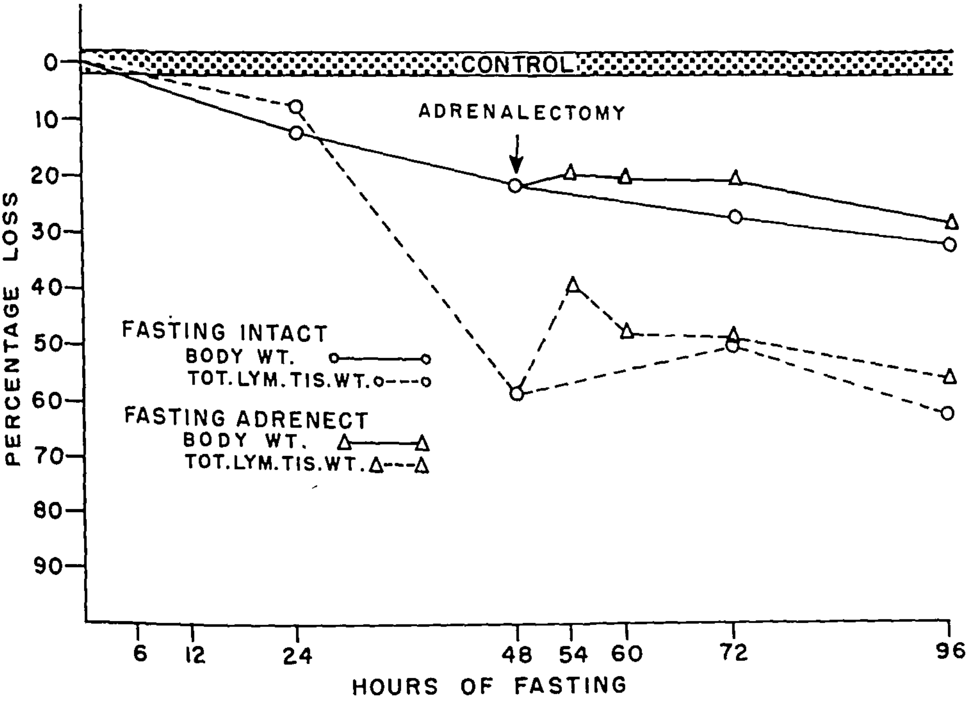

FIG. 8. Per cent loss of total lymphatic tissue weight and body weight of intact mice fasted 96 hours and of intact mice fasted 48 hours, adrenalectomized and continued to fast to 96 hours.

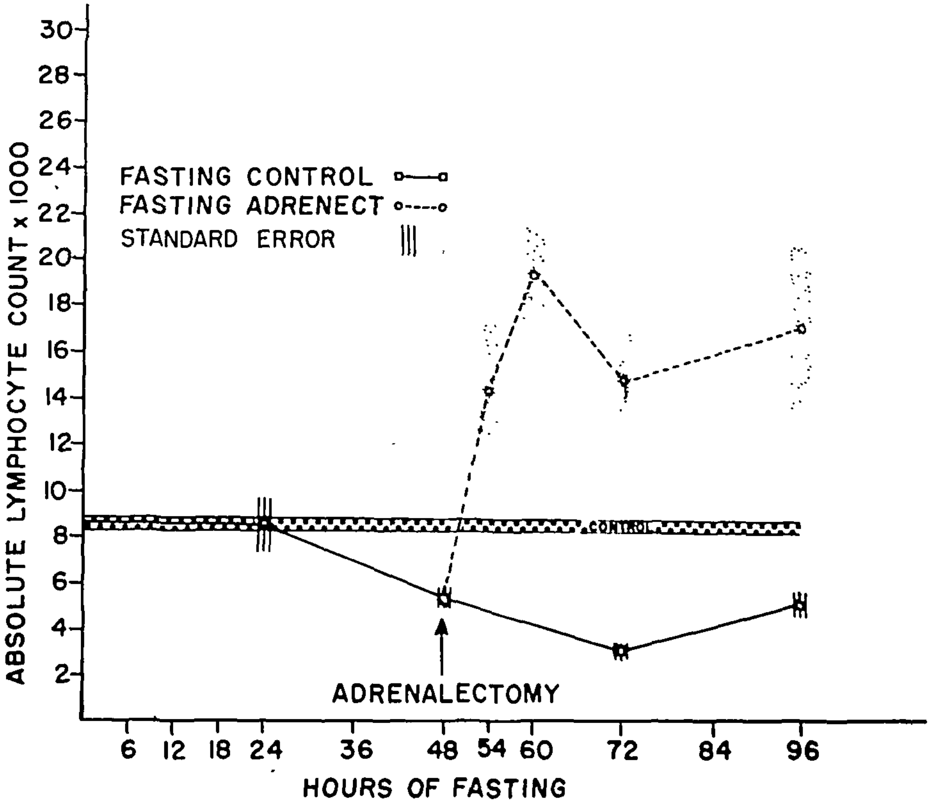

FIG. 9. Absolute lymphocyte counts of intact mice fasted 96 hours and of intact mice fasted 48 hours, adrenalectomized and continued to fast to 96 hours.

The number of mitotic figures in all lymphatic organs studied decreased during starvation. The thymus was outstanding in this respect; the mitotic index decreased from a control value of 15.6 to 0.4 at 48 hours and to 0.18 at 96 hours. When mice were adrenalectomized following a 48-hour fast and fasting was continued, a marked increase of the number of cells undergoing mitosis was found. The mitotic index of the thymus did not increase until 96 hours and then to a lesser degree than that of the spleen and mesenteric node (Table 3).

Effect of refeeding on lymphatic tissues of previously fasted intact and adrenalectomized mice. Body weights of previously fasted-intact mice increased during the first day and reached the weight range of fed animals of the same age by the third day (Fig. 1). On the other hand, the weight of the total lymphatic tissue sample did not begin to increase significantly until the fifth day of refeeding. A rapid increase in weight was then found so that by the tenth day the total lymphatic tissue weight was considered to be within the range of the total lymphatic tissue weight of intact-fed mice of the same age (Fig. 1).

Individual lymphatic organs differed in response to refeeding. Thymi of intact animals did not begin reconstitution until the sixth day of refeeding at which time a rapid gain in weight was found which attained the level characteristic of fed mice of the same age by the tenth day (Fig. 2). The spleen responded earlier and was found to return to its control range by the fifth day (Fig. 3). Although the weights of mesenteric nodes fluctuated more than those of other lymphatic organs during the entire refeeding period, weights within the range for fed-intact mice were attained by the fifth day (Fig. 4). The combined inguinal and axillary node weights were found to return rapidly to normal values by the second day of refeeding (Fig. 5).

In contrast to the intact mice which regained their body weight by the third day of refeeding, the body weight of the refed-adrenalectomized group did not return to pre-fasting levels by the end of the 8-day period of study (Fig. 1). However, the total lymphatic tissue weight was found to be increased the second day and approached normal values by approximately the sixth day (Fig. 1). In addition, in the adrenalectomized animals, no individual lymphatic organ differences were found with respect to rate of reconstitution following refeeding (Figs. 2, 3, 4, 5). The gain in total lymphatic tissue weight began almost immediately upon refeeding and continued to increase in a linear fashion. Thus, the complete reconstitution of the weight of lymphatic tissue of these animals occurred much sooner than did that of intact-refed mice (Fig. 1).

The nitrogen content per unit of lymphatic tissue weight remained the same in both intact and adrenalectomized mice during the refeeding period (Fig. 6).

Absolute numbers of lymphocytes of intact mice were found to be within the normal range for intact-fed animals by the third day of refeeding. However, in adrenalectomized mice an absolute lymphocytosis (attained within 48 hours) was noted throughout the refeeding period (Table 2 and Fig. 7). Absolute numbers of eosinophils of intact mice remained at low levels until the fourth day of refeeding; the eosinophils of adrenalectomized mice increased from a slight eosinopenia (79/cmm.) on the second day to a marked eosinophilic level (1285/cmm.) by the eighth day of refeeding (Table 2).

DISCUSSION

A classical method of inducing lymphatic tissue involution has been that of inanition and has been described extensively (Jolly, 1914; Hayashi, 1924; Jackson, 1925). Jolly (1914), in studying organ weight loss during inanition, demonstrated a loss in weight of lymphatic tissue which far exceeded the loss of body weight. Several investigators (Dustin, 1923; Undritz, 1924; Jackson, 1925; Gaetano, 1928) have reported histological of adrenalectomized-refed mice reconstituted more rapidly than the body weight. Individual differences in rate of lymphatic organ reconstitution were abolished by adrenalectomy.

The nitrogen content per unit weight of lymphatic tissue remained the same in both intact and adrenalectomized mice during fasting and refeeding.

These data support the suggestion that lymphatic tissue protein is made available for metabolic use by adrenocortical mediation. It is also suggested that the fasting-refeeding method of study can be used as a method for the bio-assay of compounds and/or nutritional factors which produce either involution or growth of lymphatic tissue.

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THE INFLUENCE OF THE LIVER UPON ESTROGEN-PROTEIN BINDING *IN VITRO*[1]

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THE liver has long been regarded as the major site of inactivation of the estrogens and other steroid hormones (*cf.*, Pincus and Pearlman, 1943; Pearlman, 1948). Recent evidence has, however, suggested that this organ may perform a dual role in estrogen metabolism, functioning both in the "activation" and inactivation of these compounds (Roberts and Szego, 1947). The mechanism of this activation may involve the elaboration by hepatic tissue of the circulating estrogen-protein complex, *estroprotein* (Roberts and Szego, 1946; Szego and Roberts, 1946).

Investigations of the capacity of surviving liver and other tissues to promote the binding of estrogen to the serum proteins have been made possible by the availability of estrone-16 C^{14} of high specific activity[2] (*cf.*, Heard *et al.*, 1951). The low order of magnitude of the substrate concentration in the present experiments is in sharp contrast to that employed by previous investigators, and may be considered a closer approximation to the physiological levels likely to be encountered *in vivo*. In addition, the choice of serum as the incubation medium represents a departure from previously reported studies in which buffered salt solutions have been utilized. In the course of the present investigation, it was found that rat hepatic tissue was capable of catalyzing the conjugation of estrone or its metabolic products with the proteins of the serum medium. This binding was accentuated in the presence of actively regenerating liver. Surviving spleen, kidney and uterus were completely inactive in this regard.

MATERIALS AND METHODS

The incubation studies were uniformly conducted in rat serum containing estrone-16 C^{14}. The animals supplying the serum and tissues for these studies were female Sprague-Dawley rats, aged 4 to 6 months, which had been maintained on Rockland

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[2] Professor R. D. H. Heard generously placed at the disposal of the investigator a sample of pure crystalline estrone-16 C^{14} prepared in his laboratories. This compound is now being distributed by the Charles E. Frosst Co., Montreal.

radioactivity in all ether extracts. That obtained with the serum proteins was similarly used for correcting radioactivity measurements on the aqueous residues. The two correction curves were of a similar order of magnitude. The majority of the determinations were carried out on infinitely thick samples (i.e., 4–5 mg./cm.^{2}).

RESULTS AND DISCUSSION

The recovery of C^{14} activity in the various fractions of the incubation medium and of normal and regenerating liver tissue is indicated in Table 1. It will be observed that the fractionation scheme employed permitted the recovery of an average of 88% of the total radioactivity added. This was the case even though respiratory $C^{14}O_2$, which may have been formed during the course of the incubation, was not measured. In addition, neither the serum proteins nor the supernatant portion of the medium was analyzed for possible residual radioactivity following separation of the fractions indicated. Finally, certain fractions normally containing up to 10% of the added activity were not analyzed in some of the experiments (Table 1). In view of these factors, the over-all recovery would appear to have been essentially complete.

It will be noted that neither the presence of liver tissue, nor variation in the time of incubation, had a consistent effect upon the over-all recovery of C^{14} activity. These observations do not rule out some degradation of the original steroid under these conditions, since a significant degree of radioactivity was noted in the aqueous back-wash fractions of a number of ether the extracts, particularly those derived from the serum proteins (Table 1; B, E). The radioactivity present in this water-soluble material appeared directly related to the radioactivity in the ether extract itself (*cf.*, A, D), and in some cases even exceeded the latter. The radioactivity of serum fraction E (possibly representing estrone breakdown products) was negligible in the experiments with spleen, kidney, and uterus but accounted for as much as 10% of the added C^{14} in incubation experiments with regenerating liver. It should be noted, however, that two of the samples of serum incubated 1 hour without tissue also exhibited considerable activity in this fraction, notwithstanding the fact that the activity in the corresponding ether extracts was negligible.

Although surviving liver had no detectable effect on the over-all recovery of added C^{14}, the distribution of isotope among the fractions of the incubation medium was markedly altered in the presence of this tissue. The bulk of the activity was present, apparently unmetabolized, in the serum supernatant ether fraction (A) in all experiments. The presence of both control and regenerating liver consistently diminished the proportion of activity in this fraction, in a degree roughly proportional to the time of incubation. The activity disappearing from this fraction was found in the

TABLE 1. THE INFLUENCE OF SURVIVING LIVER TISSUE ON THE DISTRIBUTION* OF C^{14} ACTIVITY FOLLOWING INCUBATION OF ESTRONE-16 C^{14} IN A SERUM MEDIUM

Hours of incub.	Serum supernatant			Serum proteins				Liver				Grand total
	Ether extract (A)	H_2O back-wash (B)	Total	Ether soluble (mild hydrolysis) (C)	Ether soluble (strong hydrolysis) (D)	H_2O back-wash (E)	Total	Ether soluble (mild hydrolysis) (F)	Aqueous Residue (G)	Ether soluble (strong hydrolysis) (H)	"Total"[2] (F+G)	
Serum Control												
0	88.43	1.25	89.68	0.23	0.21	0.32	0.76					90.44
	84.31	1.54	85.85	0.81	0.09	0.05	0.95					86.80
½	121.57	0.0	121.57	0.56	0.05	0.23	0.84					122.41.
1	77.35	0.05	77.40	0.15	—	—	>0.15					>77.55
	78.33	—	>78.33	0.26	—	—	>0.26					>78.59
	84.80	—	>84.80	0.21	0.26	3.30	3.77					>88.57
	85.10	—	>85.10	0.32	0.21	2.78	3.31					>88.41
	71.08	2.74	73.82	0.59	0.15	—	>0.74					>74.56
2	97.84	0.61	98.45	0.93	0.15	—	>1.08					>99.53
	87.45	—	>87.45	0.92	0.07	0.06	1.05					88.50
	120.49	0.13	120.62	0.46	0.12	—	>0.58					>121.20
Normal Liver												
0	75.88	0.77	76.65	0.14	0.05	0.12	0.31	4.57	0.81	0.06	5.38	82.34
	67.55	1.70	69.25	0.61	0.12	0.13	0.86	2.64	2.14	0.25	4.78	74.89
½	63.63	1.00	64.63	0.32	0.24	0.13	0.69	8.80	2.45	0.56	11.25	76.57
1	55.88	0.34	56.22	0.28	— (8.05)[1]	—	>8.33	6.63	6.02	—	>12.65	>77.20
	66.67	—	>66.67	0.36	—	—	>0.36	4.41	5.44	—	>9.85	>76.88
	82.84	—	>82.84	0.32	1.02	—	>1.34	12.00	4.17	0.53	16.17	>100.35
	65.59	—	>65.59	0.24	1.00	3.21	4.45	11.10	5.68	0.57	16.78	>86.82
	83.04	1.03	84.07	0.35	1.02	1.02	2.39	8.93	2.38	1.02	11.31	97.77
2	81.18	0.75	81.93	0.92	2.40	—	>3.32	8.76	3.53	0.52	12.29	>97.54
	46.86	1.24	48.10	0.90	1.70	5.30	7.90	4.56	8.29	0.67	12.85	68.85
	64.20	3.50	67.70	0.60	4.27	5.48	10.35	—	—	—	—	—
Regenerating Liver												
0	94.02	0.16	94.18	0.25	0.26	—	>0.51	6.86	—	0.21	>6.86	>101.55
	80.10	0.31	80.41	0.14	0.0	—	>0.14	3.61	1.48	0.0	5.09	>85.64
½	89.22	0.65	89.87	0.33	0.65	—	>0.98	8.96	4.35	1.36	13.31	>104.16
	78.33	0.51	78.84	0.33	0.77	—	>1.10	10.60	4.35	0.98	14.95	>94.89
1	55.29	0.37	55.66	0.35	1.59	—	>1.94	8.13	6.53	1.85	14.66	>72.26
	67.65	0.75	68.40	0.34	2.94	—	>3.28	8.30	5.79	1.44	14.09	>85.77
	59.70	1.25	60.95	0.32	2.10	1.78	4.20	—	—	—	—	—
	42.30	4.48	46.78	0.39	2.22	3.72	6.33	—	—	—	—	—
2	66.28	0.74	67.02	0.66	7.50	—	>8.16	7.11	8.24	1.38	15.35	>90.53
	67.06	1.59	68.65	0.57	7.49	—	>8.06	5.04	4.07	1.73	9.11	>85.82
	50.20	0.64	50.84	0.61	4.88	—	>5.49	4.51	5.96	1.11	10.47	>66.80
	59.50	0.39	59.89	0.48	5.67	7.87	14.02	—	—	—	—	—
	42.70	4.14	46.84	0.62	6.01	10.45	17.08	—	—	—	—	—

* For fractions designated, see text and Figure 1. Values indicated are per cent of added C^{14} recovered (of 10,200 cpm$\cong$5.82 μg. added per 3 ml. *total medium at 0 time*).

[1] Aqueous residue from first ether extraction (mild hydrolysis).

[2] "Total", liver represents, in part, adsorbed material from serum medium (present in ether-soluble fraction after mild hydrolysis). See text.

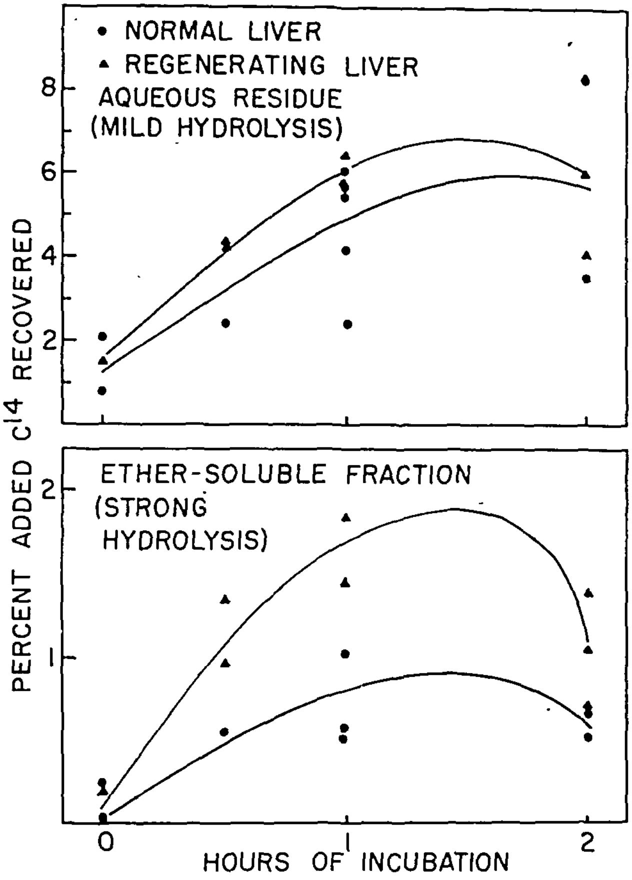

FIG. 2a,b. Recovery of C^{14} in liver tissue fractions following incubation with estrone-16 C^{14} in a serum medium. Figure 2a (upper chart), represents fraction G (cf., Fig. 1; Table 1). Figure 2b (lower chart), represents fraction H. Circles, normal liver; triangles, regenerating liver obtained 48 hours after partial hepatectomy.

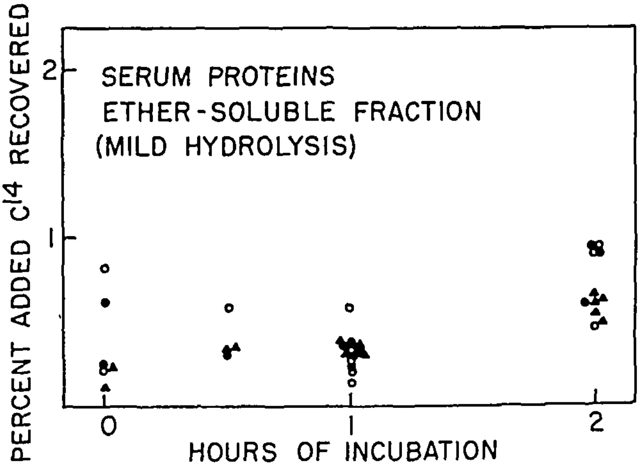

FIG. 3. Recovery of C^{14} in the ether-soluble fraction of the serum proteins following mild alkaline hydrolysis (fraction C). Open circles, serum incubated with estrone-16 C^{14} in the absence of liver; closed circles, normal liver; triangles, regenerating liver.

proteins of the medium and in the liver tissue itself. The association of C^{14} with normal and regenerating liver tissue during incubation is shown in Figure 2. Figure 2a is a schematic representation of the radioactivity remaining associated with liver components (fraction G), following preliminary mild hydrolysis and removal of ether-soluble material (F). It should be pointed out that the C^{14} activity of the latter was thought to represent, in part, material adsorbed on the tissue and perhaps rightfully to be considered derived from the serum medium. Figure 2a reveals that significant activity was present in the aqueous residue of the liver under these conditions. This activity appeared to increase with time of incubation and was higher in the presence of regenerating liver than normal liver. About one-third of the activity in this fraction could be released as ether-soluble substances after strong acid hydrolysis (Fig. 2b). In the latter instance, unincubated control samples were essentially inactive. An apparent maximum degree of binding was observed after 1½ hours of incubation.

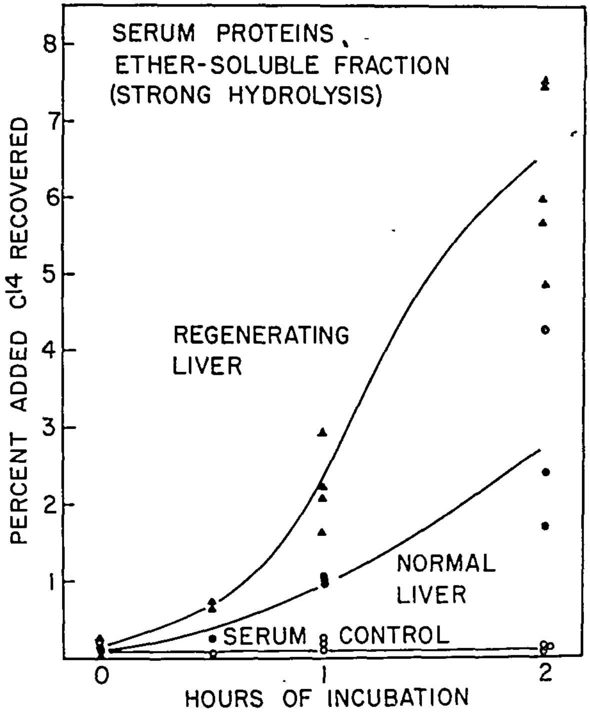

FIG. 4. Recovery of C^{14} in the ether-soluble fraction of the serum proteins following strong acid hydrolysis (fraction D). Notations as in Figure 3.

When the proteins of the serum medium were subjected to mild alkaline hydrolysis, relatively small amounts of radioactivity were liberated as ether-soluble material (fraction C). Figure 3 indicates that no significant differences occurred in the C^{14} activity of this fraction of the proteins of rat serum incubated alone for 0 to 2 hours, or incubated with normal or rapidly regenerating liver for these same periods of time. It would appear that the inconsequential amount of estrogen or its metabolic products present in the ether-soluble fraction after mild hydrolysis of the serum proteins was not an index of the estrogen-metabolizing activities of the liver.

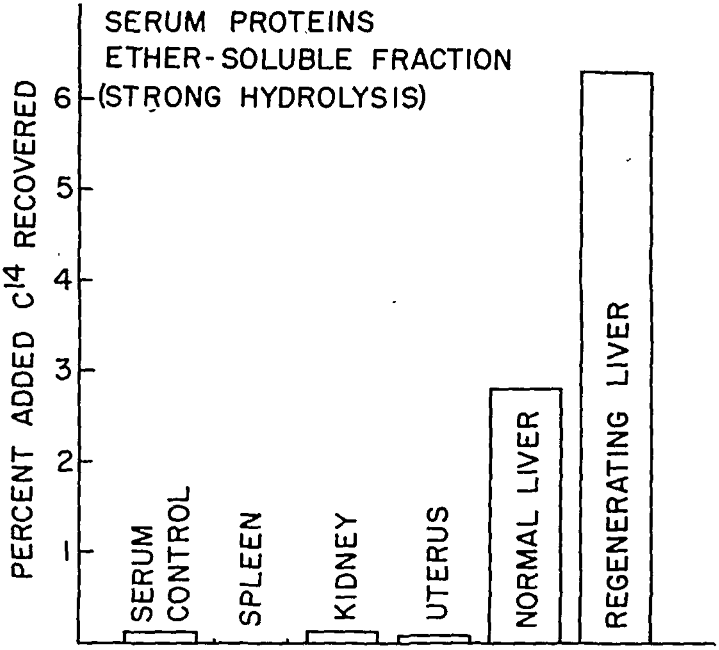

FIG. 5. Incorporation of C^{14} into the serum proteins after incubation of estrone-16 C^{14} with various tissues. The ether-soluble fraction (D) following strong acid hydrolysis is shown, as in Figure 4. All incubations were carried out for 2 hours. The height of the bars for serum control, normal and regenerating liver samples represents an average of 3, 3 and 5 samples, respectively. The values for the other tissues are the result of single determinations.

In contrast, secondary strong acid hydrolysis of the aqueous residue remaining after ether extraction of the mild alkaline digest liberated significant quantities of lipide-soluble C^{14} activity (fraction D). The radioactivity in this fraction varied significantly with the nature of the tissue and the length of the incubation period (Fig. 4, 5). This radioactive material presumably represented estrone or its metabolic products in firm combination with the serum proteins. Insignificant amounts of firmly bound radioactivity were present in the proteins of serum which had been incubated alone for 0 to 2 hours. In the presence of normal hepatic tissue, this firmly bound material increased markedly during the 2-hour incubation period. Regenerating liver, which is known to be hyperactive in many of its metabolic characteristics (*cf.*, Szego and Roberts, 1949), was extremely active in binding estrone or its metabolic products to the serum proteins *in vitro*. The capacity of surviving liver to bind radioactive material derived from estrone-16 C^{14} to the serum proteins appeared to be highly specific for this tissue. It will be observed in Figure 5 that spleen, kidney, and uterus of the rat were completely inactive in this respect.

These observations suggest that protein-binding of estrogen or its metabolic products is a physiological process, presumably catalyzed by a specific enzyme system found in liver but not in spleen, uterus, or kidney. The nature of the substances which become firmly attached to the serum proteins during incubation with surviving hepatic tissue is not definitely known. A lipide material is indicated by the solubility properties. The fact that a relatively large fraction of the original estrogen added was recovered in this form suggests that the structure may still be steroidal. That at least a portion of this material was still estrogenic was indicated by preliminary results on bioassay of this fraction. Only when liver tissue had been present in the original incubation did fraction D exhibit appreciable estrogenic activity. Other chemical properties indicate that this material was probably bound to the serum proteins in an esterified (or glucuronide) form, and, in fact, the major role of the liver in facilitating estrogen-protein binding may be by virtue of its ability to catalyze glucuronide formation (*cf.*, Crépy, 1947). On the basis of available evidence, *estroprotein*, the natural circulating estrogen, appears to be constituted of estrogen linked to serum protein in this esterified (or glucuronide) form (Roberts and Szego, 1946). The results of the present study are consistent with the hypothesis that the liver may be the site of formation of this *estroprotein.*

SUMMARY

Estrone-16 C^{14} was incubated in the presence of surviving rat tissues in homologous serum for periods varying from 0 to 2 hours. The distribution of radioactivity was studied in various fractions of the medium and tissues. Approximately 88% of the activity added could be recovered in these fractions. Normal liver tissue was capable of enhancing the binding of estrone or its metabolic products to the proteins of the serum medium. The protein-bound C^{14} activity was released in an ether-soluble form only after partial hydrolysis of the proteins. Regenerating liver was even more active in promoting binding of the steroid to the serum proteins. Spleen, kidney, and uterus were completely inactive in this regard. The relation of these observations to the problem of *estroprotein* formation *in vivo* is discussed.

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CHEMICAL TRANSFORMATION OF STEROIDS BY ADRENAL PERFUSION: PERFUSION METHODS[1]

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INTRODUCTION

STUDIES were undertaken in 1944 to discover conditions requisite for the maintenance of corticosteroid biosynthesis in isolated, perfused adrenal glands. In 1949, having developed a perfusion apparatus (Hechter, 1948) and methods for extracting and estimating corticosteroids in blood, it was possible to demonstrate that perfused beef or sheep adrenals respond to adrenocorticotrophic hormone (ACTH) by a marked increase in the rate of corticosteroidogenesis (Hechter, 1949). Possessing an active adrenal preparation, studies were then undertaken to evaluate the enzymatic capacity of perfused adrenals to modify steroids. In the course of these studies using 11-desoxycorticosterone (DOC), suggestive evidence was obtained that the gland possessed a system for directly introducing an oxygen function at C-11 of the steroid nucleus.

A few months later the dramatic therapeutic effects of cortisone in rheumatoid arthritis were reported by Hench, Kendall and their associates (1949). Since our findings represented a possible mode for the introduction of a C-11 oxygen function into the steroid nucleus, the work on adrenal perfusion was considerably expanded. This rapidly led to the definitive chemical characterization of corticosterone (Hechter *et al.*, 1949) as the DOC transformation product responsible for the glycogenic activity previously noted in adrenal perfusates. Further studies were then under-

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taken to isolate and characterize the transformation products resulting from the perfusion of a variety of C-19 and C-21 steroids through adrenals. Some of these findings have been discussed in a review in which it was not possible to give detailed descriptions (Hechter *et al.*, 1951).

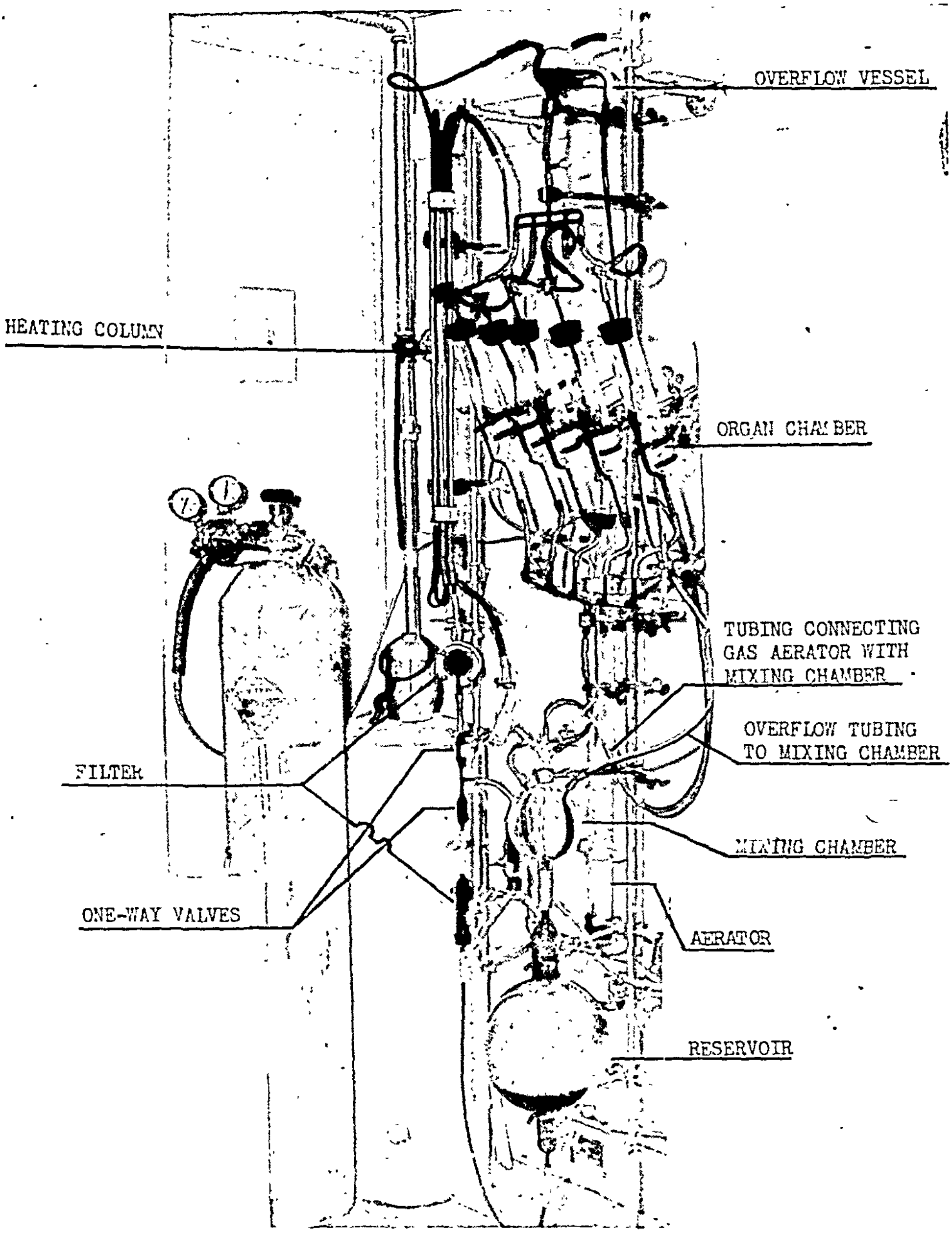
This paper is the first of a series on steroid transformations induced by isolated, perfused bovine adrenal glands and describes in detail methods of adrenal perfusion. The development of the adrenal perfusion technic to its present point was carried out primarily by studying the conversion of DOC to corticosterone; thus the data to be presented will deal mainly with the 11-bio-oxygenation system using DOC as a substrate.

METHODS

Technic of arterial perfusion of bovine glands: In the initial phase of this study, the methods developed originally for the demonstration of ACTH-induced corticosteroidogenesis (Hechter, 1949) were employed. Bovine glands were obtained at a local slaughter house. The animals available were mainly cows which for various reasons had gone off milk or heifers which had failed to breed. In a few cases, bull adrenals were obtained. It should be emphasized that the adrenals employed were derived from animals of variable age and weight, some with minor bovine diseases, whose previous nutritional regime may be safely assumed to be quite variable.

The glands were obtained after the animals had been stunned, bled out, skinned and eviscerated, approximately 15 to 30 minutes after exsanguination. With the carcass hanging on the dressing rack, one adrenal, usually the left (which is kidney-shaped in contrast to the crescent-shaped right adrenal) was removed within 1–2 minutes with all the adjacent periadrenal connective tissue, together with the abdominal aorta and vena cava. Initially, attempts were made to perfuse the gland through one of the several adrenal arteries. However, the adrenal vascular supply in bovines was found to be so highly variable that this technic was not consistently successful. Thereafter, for arterial perfusion, glands were perfused through the aorta as follows: A large cannula was inserted through one end of the abdominal aorta; a glass plug was tied into the other end distal to all adrenal vessels and all of the large extra-adrenal arteries were ligated. Particular care was taken not to disturb the fat and connective tissue surrounding the gland. The vena cava was slit open and the principal adrenal vein of the gland (in some cases there are 1 or 2 additional small veins) was cannulated, the wall of the vena cava being used as a flap to tie the venous cannula in place. The remainder of the vena cava was then carefully dissected away and the preparation was thoroughly perfused with saline or Tyrode's solution (20–40 min. after obtaining the gland) to remove all residual blood and to detect major leaks, which were then tied off. This preparation of the adrenal gland was carried out in the slaughterhouse. The glands were then taken to the laboratory, about 25 minutes away. Initially, during this interval before the glands were placed in the apparatus, they were perfused with chilled (5° C.) plasma by gravity at a pressure of 70 mm. Hg. Later it was discovered that equally good results were obtained when the preparation was transported without perfusion, but merely covered with cold Ringer's solution.

Preparation of blood for perfusion: Beef blood was collected non-aseptically at a local slaughterhouse. Large-mouthed gallon bottles containing 600 ml. of glucose-citrate solution (2.06% sodium citrate, 0.78% citric acid, and 5.0% glucose) were used for blood



Perfusion apparatus

collection from the severed neck vessels of stunned cattle. After collection, the blood was brought to the laboratory and 100,000 units of penicillin G and 500 mg. of streptomycin sulfate per liter were added to inhibit bacterial growth and the blood was stored at 2–5° C. for 1 to 7 days prior to being used for perfusion.

On the day of the experiment, the refrigerated blood was filtered first through 6–7 layers of muslin, then through a nylon stocking filter (Fenwal). This treatment removed gross clots, agglutinated red cells as well as certain scums of an amorphous character which develop in bovine blood collected and stored under our conditions. The blood was then circulated through a heterologous liver used as a biological filter designed to remove small thrombi and foreign material which might have passed through the previous filters. This preliminary perfusion treatment, in addition, removes vasoconstrictor substances present in stored blood or plasma. Instead of using homologous lung as in previous work (Hechter, 1948), rat liver was employed for this purpose as follows: The filtered blood was added to the perfusion apparatus, mounted in a 38° C. incubator room, and permitted to circulate. While the blood was being warmed and oxygenated, an etherized rat weighing 200–300 g. was exsanguinated by severing the femoral arteries and veins. The chest was then opened and a cannula was inserted through the right auricle into the thoracic vena cava directed toward the liver. The residual blood in the liver was then rapidly washed out by perfusion with physiological saline at pressures not exceeding 40 mm. Hg. The abdominal vena cava below the liver was next tied off and the liver was then removed from the animal. With this procedure. the portal vein and hepatic artery are left open and the circulation proceeds "backwards" from the vena cava→hepatic vein→portal vein (mainly) and hepatic artery. The liver was then inserted into the organ chamber of the apparatus and perfused with the medium to be used for subsequent perfusion through cattle glands. Rat livers were perfused either with pulsatile or non-pulsatile pressure (in either case the mean perfusion pressures were 30–45 mm. Hg.), depending upon whether the adrenal to be perfused subsequently was to be perfused with pulsatile or non-pulsatile pressure. Under our conditions, the blood flow through the livers was 40–60 cc. per minute and the blood was circulated for varying intervals, but at least twice through the liver preparatory to being employed for subsequent adrenal perfusion.

Addition of steroids to perfusion media: DOC and other steroids were completely dissolved in minimal volumes of propylene glycol and then added dropwise to rapidly swirled, liver-filtered perfusion blood. DOC is quite soluble in propylene glycol and was employed at a concentration of 50 mg. per ml. in this vehicle. With less soluble steroids such as 17-hydroxy DOC, Δ^5-pregnen-3β-ol-20-one, etc., it was necessary to use correspondingly more propylene glycol. Using such propylene glycol solutions of DOC, it is possible to prepare DOC suspensions in blood as high as 1 gm./liter before large particles of DOC separate (after 24–48 hours). However, this should not be interpreted to indicate that DOC concentrations lower than 1 gm./L. of blood represent true solutions.

Table 1 shows an experiment in which a beef gland was arterially perfused through the aorta with plasma containing successively increasing concentrations of DOC with a mean pulsatile perfusion pressure of 125 mm. Hg. It will be seen that upon perfusing the gland with DOC at a concentration of 30 mg./L., the adrenal blood flow is unchanged from that of the control period. When the DOC concentration was increased to 100 mg./L., there was a tendency for the blood flow to decline, and this became marked when the DOC concentration was increased to 300 mg./L. After the flow had been slowed by the latter concentration, no increase in blood flow aws observed when the DOC concentration was lowered from 300 to 30 mg. DOC/L., demonstrating that the lowering of blood flow produced by high concentrations of DOC was irreversible. It should be emphasized that none of these plasma samples containing DOC concentrations ranging from 30 to 300 mg./L. showed any turbidity. Presumably the colloidal suspension produced by 300 mg./L. DOC of plasma was so broken (possibly in the capillaries during the fluid exchanges between plasma and tissues) that mechanical blockade of about two-thirds of the vascular channels by insoluble steroid resulted.

TABLE 1. INFLUENCE OF DOC CONCENTRATION UPON ADRENAL BLOOD FLOW*

Time of perfusion, min.	Blood flow, ml./min.	Pressure, mm. Hg	Remarks
5	31	125	At 0 min., perfusion initiated with plasma, no DOC
10	36	125	
20	32	125	
40	36	125	
45	33	125	After 40′, perfusion initiated with 30 mg. DOC per L.
50	32	125	
60	31	125	
80	32	125	
85	31	125	After 80′, perfusion initiated with 100 mg. DOC per L.
90	28	125	
100	26	125	
120	27	125	
125	24	125	After 120′, perfusion initiated with 300 mg. DOC per L.
130	16	126	
140	14	125	
160	10	125	
165	8	125	After 160′, back to 30 mg. DOC per L.
170	6	125	
180	5	125	
200	7	125	At 200′, perfusion pressure increased to 200 mm. Hg.
205	19	200	
210	22	200	
200	26	200	
230	25	200	
240	23	200	

* Gland weight = 18.2 gm.

As with DOC, other steroids added to blood likewise block adrenal vascular channels at particular concentrations. In the case of each steroid, the concentration at which this occurs must be determined empirically, for it has not been possible to predict the manner in which a steroid will perfuse through an adrenal. Perhaps this depends on the nature of the metabolic products formed during adrenal perfusion, although other possibilities such as the nature and size of the steroid colloid micelles formed may be involved. For example, Δ^5-pregnen-3β-ol-20-one can be successfully perfused at 25 mg./L. whereas with concentrations of 50–100 mg./L., flow is partially blocked. Progesterone, while more soluble in propylene glycol than pregnenolone, may be perfused under our conditions without significant blockade in the range of 50–70 mg./L., but produces block at 100 mg./L. Nevertheless, Δ^4-androsten-3,17-dione, which is not significantly more soluble than progesterone in propylene glycol, has been perfused through glands at a concentration of 100 mg./L. without difficulty.

Arterial perfusion of DOC: The degree of DOC conversion into corticosterone by arterially perfused glands is shown in Table 2. In these experiments (and in those described in succeeding tables) the analytical figures for corticosteroids isolated from perfusates refer to fractions obtained by silica gel chromatography. The isolation procedure and the method of fractionation by chromatography will be described fully in a subsequent paper. Column (a), Table 2, refers to recovered crystalline starting material melting above 130°; column (b), to the crystalline product, corticosterone, melting above

160°; and column (c) to the weight in DOC equivalents of formaldehydogenic steroid (FS) present in the mother liquors of the crystallized fractions.

It will be seen from Table 2 that the rate of formation (expressed as mg. of corticosterone/hr./20 gm. gland) and the percentage yield of crystalline corticosterone vary from 3.4 mg. to 30.5 mg. and 5% to 23%, respectively, in seven experiments. The 11-oxidation of DOC occurs with plasma as well as with mixtures of blood and plasma.

The arterial perfusion method has several serious limitations which are not evident from the data of Table 2. These are: (a) The preparation of adrenals for arterial perfusion, demanding a high degree of surgical skill, could not be standardized because of variations from gland to gland. (b) A high percentage of the preparations developed serious leaks in the aorta during the course of experimentation, necessitating termination of the experiment. (c) Perfusion of glands with a variety of dyes after the experiment had been completed revealed that some areas of the gland were inadequately circulated and, in some adrenals, these ineffectively perfused areas represented one-third to one-half of the gland. (d) It was difficult to control blood flow at will, thus some preparations exhibited subnormal blood flow at high perfusion pressures, while in others, despite low perfusion pressures, the flow was relatively rapid. (e) Finally, arterially perfused preparations contained a great deal of extra-adrenal tissue (which tended to complicate any results obtained) which could not be dissected away without injury to the adrenal arterial vessels running through them.

TABLE 2. 11-HYDROXYLATION BY BOVINE GLANDS PERFUSED *via* AORTA

Expt. No.	Perfusion conditions						Perfusate analysis			Rate†
							Crystalline			
	Gland wt., gm.	DOC input, mg./L.	Medium	Mean arterial pressure, mm. Hg.	Mean blood flow, ml./min.	Perfusion period, min.	DOC (a) %	Cort. (b) %	Non-cryst. F.S., %	
1	18.5	30	Plasma	125	27	64	38	8	23	4.3
2	20	50	Plasma	120	7	222	24	16	20	3.4
3	24	50	Bl. Mixture*	100	8	167	22	23	17	4.6
4	12	70	Plasma	100	21	48	23	10	35	14.7
5	12	70	Bl. Mixture*	110	27	112	39	8	15	15.1
6	13	100	Plasma	190	22	340	32	15	11	30.5
7	19	100	Plasma	130	23	44	41	5	16	7.3

* Refers to equal volumes of plasma and whole blood.
† mg. of corticosterone per hour of perfusion per 20 gm. gland.

Accordingly an attempt was made to develop a simpler method adaptable to routine standardization whereby these difficulties could be reduced or improved.

Venous perfusion: Consideration of the circulation of the adrenal suggested that venous perfusion might remedy some of the above problems. Since there is a single large adrenal vein which empties directly into the vena cava, this vessel could easily be cannulated within 1–2 minutes. By perfusing *via* the vein, fluid was delivered first into the venous lumen, then through the medulla, next the cortex, and finally out the innumerable adrenal arteries at the surface of the gland, a reversal of the normal process. In this type of preparation, all of the periadrenal fat and connective tissue were superfluous and could be rapidly dissected away. The entire preparation required no more than 5 minutes and little surgical skill. Venous perfusion of glands had previously proved to be successful in a preliminary study (Hechter, 1949) and accordingly a systematic investigation of venous perfusion was undertaken.

It was found that when glands were cannulated for venous entry of the perfusion medium the blood flow was exceedingly slow despite utilization of mean pulsatile perfusion pressures as high as 200 mm. Hg. In Table 3 are shown the data of 6 experiments using glands perfused via the adrenal vein. It will be seen that such preparations 11-

oxygenate DOC when blood was present in the perfusion medium (Expts. 4, 5 & 6), but in marked contrast to the previous results (*cf.* Table 1) no detectable corticosterone was formed when DOC was perfused in plasma as the medium (Expts. 1, 2, & 3).

This difference between media in venous preparations became explicable as the result of visual inspection of glands perfused with whole blood. Following each pulsatile stroke (which pumped fully oxygenated blood into the central venous lumen distending the gland) a pool of dark deoxygenated blood passed back upon recoil from the gland lumen into the glass cannula. Soon a considerable portion of the cannula was filled with deoxygenated blood which surged back and forth as the pulsatile perfusion continued. These observations suggested that the blood flow obtained by venous perfusion of glands was so slow that the oxygen dissolved in the medium was depleted by the adrenal medullary tissues. Thus it appeared likely that with plasma, whose oxygen capacity is limited, the perfusion medium entering the cortical layers was so completely deoxygenated that the bio-oxygenation of DOC could not occur. With blood, however, whose oxygen-carrying capacity is high, sufficient oxygen was retained in the medium to permit the oxidative process to function.

TABLE 3. 11-HYDROXYLATION BY BOVINE GLANDS PERFUSED *via* ADRENAL VEIN

Expt. No.	DOC input, mg./L.	Gland wt., gm.	Medium	Mean pressure, mm. Hg.	Mean blood flow, ml./min.	Perfusion period, min.	Crystalline		Non-cryst. F.S.,	Rate†
							DOC %	Cort. %		
1	40	18	Plasma	150	6.8	338	33	0	25	0
2	50	22	Plasma	100	10.0	200	42	0	12	0
3	100	20	Plasma	100	6.1	155	33	0	12	0
4	50	20	Bl. mixture*	150	7.1	326	4	35	31	7.5
5	70	13	Bl. mixture*	120	11.6	222	6	36	18	27.0
6	100	20	Bl. mixture*	100	10.7	89	30	10	20	6.4

* Refers to equal volumes of plasma and whole blood.
† Refers to mg. of corticosterone per hour of perfusion per 20 gm. gland.

From hemodynamic consideration, it appeared most likely that the slow blood flow with venous-perfused glands resulted from a constriction of the arterioles and small arteries in the cortical layers near and at the surface of the gland. Attempts to increase the flow by means of a variety of vasodilators and by sympatholytic agents were, however, without significant influence.

Venous perfusion: Lacerated glands—pulsatile pressure.—A method of overcoming this difficulty of flow in venous-cannulated preparations presented itself as the result of an accident. In two experiments when the mean pulsatile pressure was increased to about 200 mm. Hg, the gland burst after a relatively short period and blood containing DOC was pumped rather rapidly through the leaks which developed. Considering that the major portion of DOC escaped through the leak without passing through adrenal tissue, it was surprising to find rather high concentrations of corticosterone upon workup of perfusate samples from these burst glands. This observation led us to lacerate with a razor blade the outside surface of the gland to a depth of 2–3 mm. on the basis that such lacerations might provide a by-pass for the escape of blood which had circulated through the adrenal tissue. Table 4 illustrates the relationship between the number of lacerations placed on the gland surface and the adrenal blood flow under a constant pressure head of 90 cm. of H_2O. It will be seen that the flow through the gland (at constant perfusion pressure) initially rises rapidly and then tends to level off as the number of lacerations is increased progressively. Using the venous-lacerated type of preparation, it was soon learned that the steroid conversion rates were higher than those achieved by our previous procedures.

Venous perfusion: Lacerated glands—non-pulsatile pressure.—During the course of

TABLE 4. THE INFLUENCE OF THE NUMBER OF LACERATIONS UPON FLOW THROUGH A VENOUS CANNULATED GLAND*

Lacerations		Flow, ml./min./gm.
Number	Total length, cm.	
0	—	0.11
1	6.1	0.43
2	11.5	1.02
3	16.0	1.43
4	24.0	1.72
5	30.0	2.10
6	35.2	2.42

* A gland weighing 18.7 gm. was perfused with Tyrode's solution at a constant pressure of 90 cm. H_2O. Length of individual lacerations were measured with flexible mm. rule.

perfusing lacerated glands via the vein with pulsatile pressures, it was noted that many glands burst during the course of several hours of perfusion and it was not possible to evaluate blood flow rates. It seemed likely that these tears in the gland resulted from the alternate flexing and relaxing (induced by pulsatile perfusion pressures) of glands whose connective tissue capsule had been effectively destroyed by the lacerations. Accordingly, the pulsatile pressure of the perfusion apparatus was eliminated and the pump was used merely for elevating the blood for non-pulsatile perfusion under suitable hydrostatic pressure. With this technic, hundreds of lacerated glands have been perfused through the adrenal vein and in only an occasional instance has a gland developed "leaks" during perfusion periods for as long as 12 hours. Accordingly, the non-pulsatile perfusion technic became our method of choice. Table 5 illustrates the rate of 11-hydroxylation of DOC in venous-lacerated glands (perfused with pulsatile and non-pulsatile pressures). The high rates of conversion obtained should be compared with results shown in Tables 2 and 3.

TABLE 5. COMPARISON OF PULSATILE AND NON-PULSATILE PERFUSION OF LACERATED GLANDS CANNULATED *via* THE ADRENAL VEIN WITH 100 MG. DOC PER L

Expt. No.	Type	Perfusion medium		Gland wt., gm.	Mean pressure perfusion mm. Hg	Mean bl. flow, ml./min./gm.	Rate*
		% blood	% plasma				
1	Non-pulsatile	10	90	14	28	1.03	32
2	Non-pulsatile	50	50	17	30	1.20	48
3	Non-pulsatile	50	50	15	26	1.15	57
4	Non-pulsatile	100	0	22	60	1.35	48
5	Non-pulsatile	100	0	18	100	1.35	36
6	Pulsatile	10	90	22	19	1.10	48
7	Pulsatile	20	80	20	20	1.00	52
8	Pulsatile	50	50	17	32	1.32	42

* mg. of corticosterone per hour of perfusion per 20 gm. gland.

Laboratory scale production of cortisone by perfused adrenal glands: Since corticosterone, the product isolated from DOC perfusions, was at that time (spring, 1950) unavailable in other than milligram amounts from adrenal extracts, it was felt important to produce gram quantities of this corticosteroid for biological experimentation. As our objective, production of 5 to 6 gm. of corticosterone was set. A bank of individual glands was perfused with DOC in blood under conditions designed to insure a good yield of corticosterone, no attention being given to the rate of production. From our foregoing experimental work, it was decided to recirculate 200 mg. of DOC/L. of blood through a bank of 5 glands, each with its own entry tube from a manifold. By proper adjustment of stopcocks, it was possible to regulate adrenal blood flow to equalize large differences in flow rate between individual glands so that all glands would have a blood flow of about 1.5–2.0 L. per hour, independent of gland weight..

Table 6 shows the results of five consecutive experiments using 20 to 28 L. of blood. In experiment A, the perfusate was circulated once through two 5-gland banks, in the remaining runs (B to E), the perfusate was circulated 2 to 3 times through 1 such bank. The average yield of crystalline corticosterone produced under these conditions was 37%, based on the weight of the DOC recovered from the perfusate. In total, 5.63 gm. (34%) of purified corticosterone was obtained from the 16.6 gm. of desoxycorticosterone disappearing from 108 L. of perfusate.

TABLE 6. THE PRODUCTION OF CORTICOSTERONE BY BANKS OF ADRENAL GLANDS PERFUSED WITH DOC

Run No.	Perfusate vol., liters	Input DOC, gm.	Cryst. unaltered DOC, gm.	Cryst. cort., gm.	% cryst. cort.*
A	28	5.5	0.83	1.84	39
B	20	4.0	1.21	0.92	33
C	20	4.0	1.12	1.05	36
D	20	4.0	0.83	1.23	39
E	20	4.0	0.88	1.16	37

* Based on the weight of desoxycorticosterone recovered.

In considering these results, it should be emphasized that approximately half of the added steroid is unaccountable as either crystalline DOC or corticosterone by our isolation method.

DISCUSSION

An example of the perfusion method guided exclusively by biochemical principles has been given. Conditions of perfusion have been selected solely on the basis of the rates of an *in vitro* reaction, and not upon physiological considerations. Approached in this fashion, a simple technique for perfusion of bovine adrenals (as well as for the adrenal glands of sheep, pig, horse and human, the other species tested) has been developed which requires probably less skill than is necessary for the preparation of adrenal slices. Since our findings may be generally applicable to a variety of problems in organ metabolism, some aspects of these studies merit discussion.

Organ transfer from the animal to the perfusion pump: The present work demonstrates that in the case of the specific enzymatic activity studied, the C-11 hydroxylation system, it is not necessary to transfer the organ rapidly from the animal to the perfusion pump. Glands are routinely perfused which have been obtained from animals killed several hours prior to experimentation. Indeed, glands stored at 2°C. in salt solution and perfused 24 hours after the death of the animal are still capable of transforming DOC to corticosterone, albeit at a somewhat reduced rate. It should be emphasized that the stability of the C-11 "oxidase system" described is not a special case of an "exceptional" enzyme. The entire series of biochemical events involved in the formation of corticosteroid hormones and cholesterol from C-2 fragments, involving a multiplicity of enzymes coupled with the appropriate energy-yielding reactions, also proceeds in such glands perfused hours after the death of an animal (Hechter, *et al.*, 1951) (Zaffaroni *et al.*, 1951).

It should be emphasized that all glands were stored in salt solutions at or near 0°C. The temporary anoxia involved in gland storage of concern to some physiologists who believe that organs should be transferred to the artificial circulation as rapidly as possible to minimize anoxia, ignores the point that the dimensions of anoxia involve temperature as well as time. The question as to whether an organ stored near 0°C. is anoxic cannot be answered *a priori* but requires a determination of the oxygen requirements for the maintenance of the system at that temperature. It seems likely that a great variety of enzymatic processes in tissues other than the adrenal will prove to be sufficiently stable (at low temperature) to allow time for an unhurried surgical preparation of the organ for perfusion. Where a highly unstable constituent is a component of the reaction under investigation, it may be possible to overcome such deficiencies by preliminary perfusion with blood, with presumable resynthesis of the "unstable" component.

Preparation of organs for perfusion: Ordinarily, one thinks of perfusing an organ through the artery since this is the normal course of circulation. Biochemically, however, it is of no fundamental concern whether substrates are presented to cells from the venous or the arterial side of the capillary. The present study provides an illustration of this principle in the case of an organ wherein "normal" arterial perfusion is very difficult from a surgical point of view, but where the venous cannulation is simple. The difficulties in blood flow exhibited in this preparation were overcome by lacerating the cortex of the gland. While this treatment undoubtedly damages cells, quantitatively the total number of cells so injured is vanishingly small relative to such injury in tissue slices. Application of these considerations to the preparations of other organs will undoubtedly remove many of the surgical difficulties formerly associated with perfusion. Finally, it should be mentioned that organs of small laboratory animals with blood vessels too small for easy cannulation may be replaced by slaughterhouse material, allowing the use of numerous organs from a variety of species.

Perfusion media: We have previously shown that a variety of perfusion media may be employed to demonstrate 11-hydroxylation of DOC in bovine adrenals and that, of these, homologous beef blood seems to be the most satisfactory (Hechter *et al.*, 1951). This should not be taken to indicate, however, that physiological media are necessary to obtain maximal activity of a particular metabolic system. The blood used in our studies, while homologous, contained abnormally high concentrations of glucose, citrate and antibiotics. Citrated blood was originally used in our studies when it became prohibitively expensive to employ the "more physiological" anticoagulant, heparin. As it developed subsequently, citrated blood may well be superior to heparinized blood for the study of a number of adrenal corticosteroidogenic processes. The use of homologous blood in our present experiments represents an accident of convenience since both beef glands and blood were available. In other studies it has been shown that bovine blood is as effective as sheep blood in ACTH-induced steroidogenesis in sheep glands (Hechter, 1949). The use of heterologous blood in organ perfusion represents a solution of those problems which arise when the organs perfused are derived from animals which cannot easily supply adequate volumes of homologous blood. As an illustration of this point, it may be mentioned that in steroid metabolic studies of perfused rat livers carried out by one of us (O.H.), bovine blood has proven especially useful as a convenient perfusion medium on a 20 L. scale.

Role of perfusion in biochemical investigation: We have previously stated that a perfused organ may be likened to an organized collection of tissue slices wherein the circulatory apparatus is employed to permit entry of substrates into the cells and have discussed some of the factors which influence the rate of biochemical reactions in a perfused organ (Hechter, *et al.*, 1951), *e.g.* capillary hydrostatic pressure, permeability of substrate through the capillary wall, possibility of change in the number of effective vascular channels, etc. These added complexities of the perfusion technic, difficult to control quantitatively, limit the usefulness of the perfusion method.

What, then, is the place of perfusion in modern biochemical investigation? In our opinion, the present study affords one clearly useful example. Although corticosteroidogenesis has long been regarded as involving a series of transformations presumably starting from cholesterol, the metabolic pathways involved and the end products were subjects of speculation until our perfusion work provided a basis for the beginning of an understanding of the intermediaries and enzymatic systems involved (Hechter, *et al.*, 1951). The present report is concerned with one step in this process, the 11β-hydroxylation of DOC, and illustrates perfusion as a method for obtaining sufficient material to enable the definitive chemical characterization of an unknown metabolic product derived from a specific substrate. Once products of metabolic reactions are known, moreover, it becomes possible in many cases to device suitable micromethods for the study of active systems by simpler *in vitro* methods. Thus, following our demonstration of the 11-hydroxylation of DOC, confirmation of this reaction in adrenal slices, breis, and homogenates was soon accomplished (Hayano *et al.*, 1949) (Savard *et al.*, 1950) (McGinty *et al.*, 1950) (Haines, 1952). It seems clear that a detailed characterization of the enzymatic system involved will not significantly be facilitated by perfusion work but requires enzymological techniques. In similar fashion, other reactions described in the perfused adrenal have likewise been demonstrated in homogenates such as the 17- and 21-hydroxylation of steroids (Hayano and Dorfman, 1952) (Plager and Samuels, 1952), the conversation of Δ^5-3β-hydroxy to Δ^4-3-ketosteroid (Samuels *et al.*, 1951), and the condensation of C-2 fragments from acetate to corticosteroid hormones (Haines *et al.*, 1951) (Haynes *et al.*, 1952). The conversion of cholesterol to corticosteroid hormones has not as yet been achieved in simpler systems; studies involving the latter system in perfused glands will continue to be useful until this system is achieved in homogenates or slices. While the examples cited above are drawn from a single field, corticosteroid biosynthesis, we suggest that the application of the perfusion method to the study of the metabolism of a variety of biologically active substances is bound to be profitable.

Having discussed areas wherein perfusion may prove important, one general aspect of perfusion relative to other *in vitro* methods should be mentioned. With both slices and homogenates following the addition of substrate, the products of reaction accumulate. These products may either inhibit the rate of reaction or, depending upon specific conditions, may be further converted into secondary and tertiary products in a "normal" or "abnormal" pathway. Moreover, as the reaction proceeds, the level of substrate declines continuously from its original concentration. Perfusion without recirculation of the perfusate provides an *in vitro* metabolic system wherein substrates can be administered at a constant concentration during the course of experimentation and products of reaction may be removed continuously in a manner comparable to the *in vivo* situation.

SUMMARY

A description is given of the development of methods of perfusing isolated beef adrenal glands using 11-desoxycorticosterone as the substrate for the gland's 11-oxygenating system. A study of the perfusion of DOC in various media by arterial and venous perfusion has led to the use of gravity flow through lacerated beef glands *via* the adrenal vein. The limitations of the perfusion method for the study of the enzymatic systems involved in corticosteroidogenesis as well as its use as a tool in general biochemical investigation are discussed.

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β-GLUCURONIDASE STUDIES IN INBRED MICE: ANDROGENIC HORMONES AND KIDNEY AND URINARY β-GLUCURONIDASE ACTIVITY[1,2]

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INTRODUCTION

THE effects of sex hormones on tissue β-glucuronidase activity in inbred mice were reported in a previous paper (Fishman & Farmelant, 1953). Among other findings, it was established that testosterone propionate produced a marked elevation in kidney β-glucuronidase in all inbred strains which were studied. In some preliminary histochemical experiments, it appeared that the enzyme was localized in both the lumina and in the epithelial cells lining the renal tubules. This suggested that some of the androgen-stimulated kidney β-glucuronidase may be excreted in the urine. The present paper deals with experiments designed to study this possibility.

METHODS AND EXPERIMENTAL PROCEDURES

Details may be found in a previous publication (Fishman and Farmelant, 1953) concerning types and source of animals used, the nature of the hormone treatment, types of controls, method of assay of β-glucuronidase activity, etc.

The routine of urine collection was as follows. Often at two times in the day, 10 A.M. and 4 P.M. and always once each day, 4 P.M., the mice were induced to urinate into Wassermann test-tubes, by gentle pressure with the fingers on the bladder area. A 1 to 10 dilution was prepared (0.1 ml. urine+0.9 ml. H_2O) ordinarily, but 1 to 50 and 1 to 100-fold dilutions were required for the assay of β-glucuronidase-rich urines. When the amounts of urine were insufficient, specimens of two or more mice in the same group were pooled.

A period of incubation was selected in which not more than 5% of the substrate phenolphthalein glucuronic acid present in the digest was hydrolyzed (1 hour for β-glucuronidase-rich and 18 hours for β-glucuronidase-poor urines). The amount in micrograms of phenolphthalein liberated was measured (Fishman, Springer and Brunetti, 1948) and the urinary β-glucuronidase (phenolphthalein units) expressed in terms of 1 hour incu-

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bation of undiluted urine (100 ml.). No difference in urinary β-glucuronidase was found in urines, one aliquot of which was assayed immediately and another aliquot being allowed to remain stoppered in the refrigerator for as long as 3 days before assay. The possibility of contamination of the urine by β-glucuronidase-rich bacteria is considered remote on the basis of negative control experiments with toluene-preserved and nonpreserved urines. The design of the experiments is readily understood from the material presented under results.

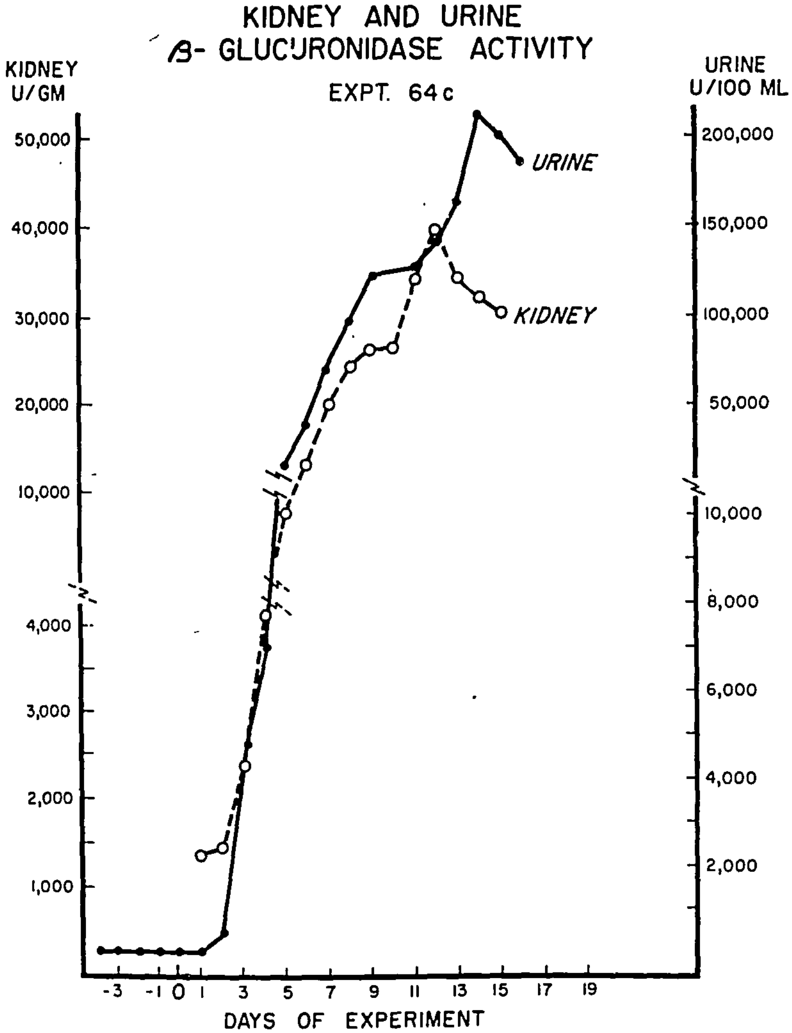

FIG. 1. Kidney and urinary β-glucuronidase activities in DBA female mice injected for 16 days with 2.5 mg. daily of testosterone (Oreton F, *Schering*, aqueous suspension). Each white circle represents toe average of β-glucuronidase values of the kidneys of 3 mice and each black circle, the β-glucuronidase activity the value of fresh pooled urine of the surviving animals at the corresponding experimental day.

TABLE 1

PART I. URINARY β-GLUCURONIDASE ACTIVITY OF FEMALE DBA MICE

Days of ℞*	Individual Mouse Values (Units/100 ml.)												
	Animal Nos.												
	1	2	3	4	5	6	7	8	9	10	11	12	Pooled Urine Data†
−7	0	0	0	0	0	0	0	0	0	0	0	0	
−6	0	0	0	0	0	0	0	0	0	0	0	0	
−3	0	0	0	0	0	0	—	—	—	—	—	—	
−2	—	0	—	—	—	—	—	0	—	0	0	0	
−1	—	0	—	0	0	0	0	0	—	0	0	—	
+3	965	603	664	664		0	0	664	664	0	0	956	
+4	2,710	2,760		4,030	1,900	2,250	3,510	4,020	3,570	4,500	2,130	4,500	
+5		5,180		9,840		5,120	9,840	12,200		15,600		9,400	
+6	21,700	10,600	11,400	22,800	18,950		24,400		27,300		18,350		
+7	20,200	11,390	9,690	27,300	25,400		21,600	25,420	22,680		20,700		26,900 (6, 10)
+8										45,800	35,500	46,500	42,600 (3, 9)
													47,100 (1, 2, 7, 8)
+9			90,000			86,000	83,000			138,000			154,000 (4, 11, 12)
+10	102,000			170,000		81,000	140,000	172,000	122,000	140,000	150,000	195,000	129,000 (2, 3, 5)
+11	187,000	105,000	123,000										180,000 (4, 5, 6, 11)
+12	150,000	100,000	130,000	134,000		100,000	134,000				144,000		214,000 (5, 9, 10, 12)
+13		92,000	140,000	220,000	140,000	120,000	282,000					209,000	203,000 (1, 9, 10)
+14		90,000					130,000						185,000 (9, 10)
													138,000 (1, 5,8)
													125,000 (6, 11)
+17		118,000	129,000		166,000	122,000							217,000 (1, 4, 9)
													200,000 (7, 8, 12)
+19	206,000	152,000	136,000	180,000		185,000		236,000	195,000	187,000	168,000	185,000	
+21			172,000			254,000							292,000 (10, 11)
													270,000 (1, 2, 4, 5)
+24	207,000	132,000	172,000						146,000		113,000		233,000 (1, 10, 12)
													147,000 (6, 8)
+25				312,000						220,000	136,000	190,000	163,000 (5, 6)
													236,000 (3, 9)

PART II. TISSUE β-GLUCURONIDASE ACTIVITY (UNITS/GM.) OF FEMALE DBA MICE

Days of ℞*	Mouse	Kidney	Liver	Spleen
26	9	32,400	6,030	4,350
26	12	45,400	7,300	4,560
27	10	36,700	6,520	5,580
27	11	38,000	7,050	6,140
28	5	44,200	4,450	4,650
28	8	43,200	5,800	3,590
56	2	50,300	6,390	5,100
56	4	42,500	—	—
60	1	58,500	6,900	5,810
60	6	57,400	6,530	4,940
	Controls			
	1	1,260	4,300	5,630
	2	1,770	3,480	5,400
	3	1,670	6,370	6,860

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* Each of 12 DBA female mice 3 months old received 2.5 mgm. of an aqueous suspension of testosterone propionate daily for 60 days. Urine was collected once daily when possible for 25 days after injections began.

† Insufficient specimens were pooled for β-glucuronidase assays. The numbers in parenthesis identify the mice supplying this urine.

RESULTS

Urinary and renal β-glucuronidase in androgen treated mice. Results of a representative experiment of this study are given in Table 1. Urinary β-glucuronidase concentration was determined in individual female DBA mice from 7 days prior to and during 25 days of daily injections of testosterone propionate. It may be noted that in the control period the urines were negative (43 specimens from 12 mice collected individually over a 6-day period). Positive indications of the excretion of β-glucuronidase were found in eleven mice by the fourth day of hormone administration. In the case of each of the 12 mice, the urinary β-glucuronidase levels continued to rise as a rule. These rose rapidly in the first days and less so in the later period of observation. Six of these mice, sacrificed at 26, 27 and 28 days exhibited renal β-glucuronidase activity much greater than normal. By 60 days of treatment, the renal β-glucuronidase was still high.

Time relationships in the androgen-induced renal and urinary β-glucuronidase response. Essentially the same experimental conditions were followed as in the experiment described in the preceding paragraph, except that groups of several mice were sacrificed at regular intervals in order to measure the kidney β-glucuronidase activity during the 16-day period of testosterone administration (Fig. 1). Both kidney and urine β-glucuronidase exhibited significant increases on the third day of the experiment and the rate of increase in enzyme activity for both was similar.

Histochemical localization of renal β-glucuronidase in androgen-stimulated mice. The method of Friedenwald and Becker (1948) utilizing the substrate, 8-OH, quinoline glucuronic acid, was applied to thin sections of kidneys taken from mice treated with androgen. Plate I illustrates the localization of the stain, representing β-glucuronidase activity, in the tubules of the cortex. This section is representative of many such from experiments on androgen-stimulated kidneys.

DISCUSSION

In attempting to evaluate the experimental evidence concerning the possible excretion in the urine of androgen-stimulated renal β-glucuronidase, it is of interest to review information concerning kidney and urinary β-glucuronidase. Two phenomena have been described; one is the sex difference of kidney β-glucuronidase noted in certain strains of inbred mice (Morrow, Carroll and Greenspan, 1951, Fishman and Farmelant, 1953) and the other is the low renal glucuronidase of C3H mice (Morrow, Greenspan and Carroll, 1949, Fishman and Farmelant, 1953). It is found in this laboratory: (1) that in strains showing sex differences (e.g. DBA, White Swiss) urines from male mice were almost always richer in β-glucuronidase than those collected from female animals and (2) that C3H mice

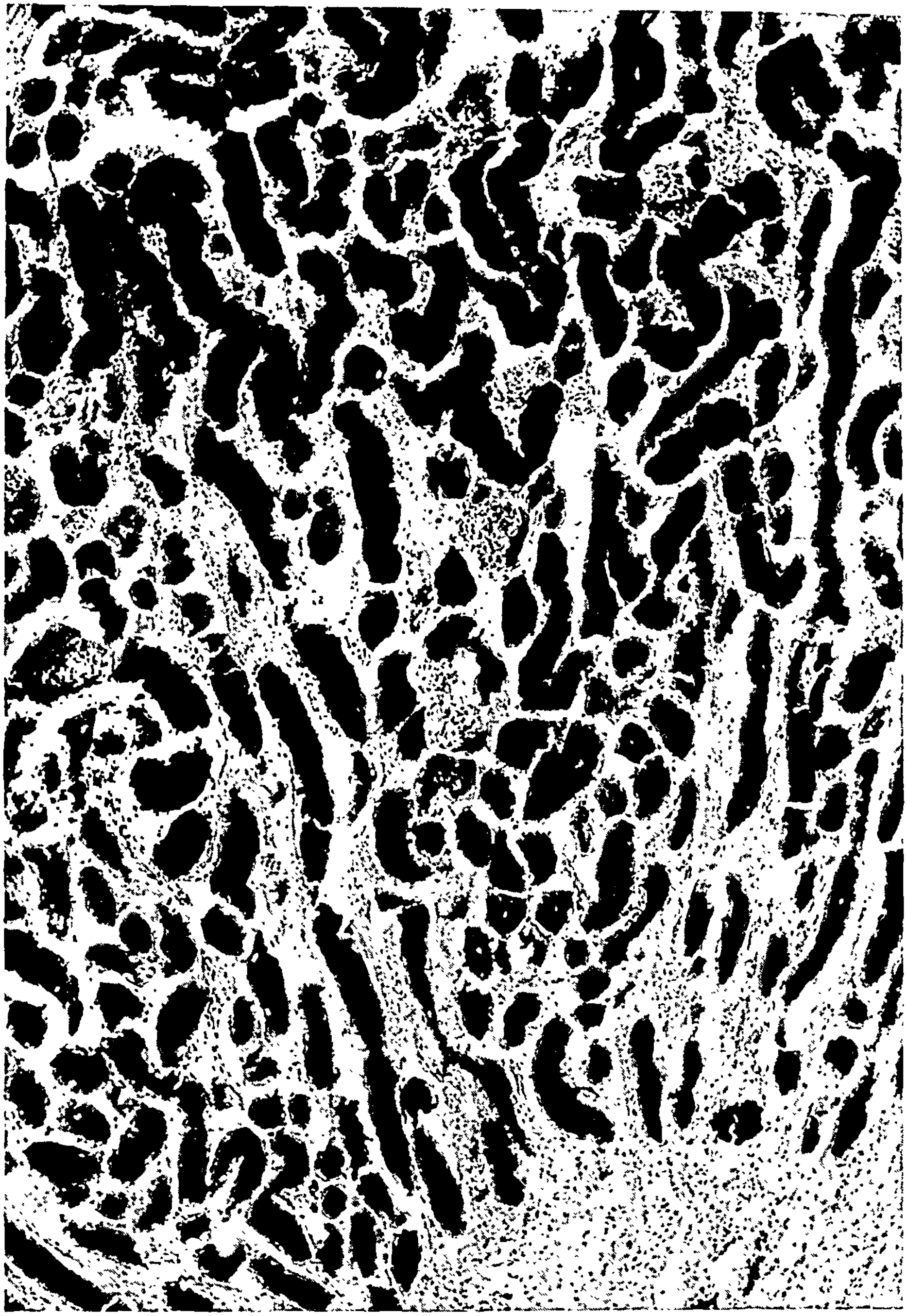

PLATE I. Localization of β-glucuronidase in the kidney of a C3H male mouse treated with testosterone-propionate for 30 days. Period of incubation with 8-OH quinoline glucuronide 18 hours. β-Glucuronidase activity 14,000 units/gram. Magnification 400×. Section is 10μ in thickness.

with very few exceptions excrete no urinary β-glucuronidase in contrast to the high β-glucuronidase mice (DBA and White Swiss mice, particularly of the male sex) (Riotton and Fishman, unpublished).

The present data demonstrate that the administration of testosterone propionate to mice induces an increase in β-glucuronidase of the renal tubules with an augmented level of this enzyme appearing in the urine. In addition to the studies in female DBA mice, qualitatively identical results were observed in male White Swiss mice given testosterone propionate or methylandrostenediol and in C_3H male mice receiving testosterone propionate.

An insight into the mechanism of the urinary excretion of β-glucuronidase in animals given androgen is gained from Figure 1 which shows the time relations in β-glucuronidase activity for kidney and urine. Thus, it is clear that β-glucuronidase in significant amounts is found in the urine not before but on the first day an increase in kidney β-glucuronidase was observed. It appears that the enzyme finds its way from the kidney tubules directly into the urine without delay and suggests a phenomenon localized to the genito-urinary apparatus.

Many histological preparations of kidneys from androgen-treated animals did not reveal pathological changes. Although the urine of DBA and other mice normally contains protein in small amount, it was possible to detect the excretion of abnormal amounts of protein in the urine of androgen-treated animals. The question of how much of this protein is β-glucuronidase remains to be answered.

A discussion of the relevance of other important considerations, such as the renotrophic effects of androgens, to the phenomena reported in this and the previous paper is being reserved for a later communication.

SUMMARY

Testosterone propionate increased by substantial amounts the excretion of β-glucuronidase in the urine of mice. This phenomenon was accompanied by the simultaneous elevation of β-glucuronidase activity in the kidney tubules. Under the conditions of the experiments, significant augmentation in both renal and urinary β-glucuronidase of androgen-treated DBA mice was observed by the third experimental day.

Acknowledgments

The technical assistance of Mr. S. Green and Miss B. Kvedar is gratefully acknowledged.

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THE INACTIVATION OF INSULIN BY LIVER SLICES OF THE RAT[1]

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THE demonstration that extracts of rat liver contain an insulin-inactivating system (insulinase) (Mirsky and Broh-Kahn, 1949) and an inhibitor of this system (insulinase-inhibitor (Mirsky, Simkin and Broh-Kahn, 1950) raises the possibility that the rate of insulin inactivation within the intact cell is determined by the balance of activity resulting from the mobilization of either of the two systems. Since the assay of the enzymatic activity of extracts may not represent the "working" activity of the cell prior to disintegration by homogenization, it was deemed essential to investigate the inactivation of insulin by relatively intact cells such as comprise surviving slices of liver.

METHODS

Fed male rats of from 150 to 200 gm. in weight were decapitated and as much blood as possible was drained from the carcass of each animal. The liver was removed and placed in cold phosphate buffer, pH 7.4. Slices of approximately 1 cm^2 in size were prepared with a Stadie-Riggs apparatus (Stadie and Riggs, 1944). After blotting gently with filter paper, the slices were weighed and the desired amount placed into a Warburg flask containing 2 ml. of the phosphate buffer in the main compartment and 3 units of insulin in 1 ml. of buffer in the side-arm. When insulinase-inhibitor was employed, it was dissolved in the buffer containing the insulin. After gassing for 5 minutes with 95% oxygen+5% CO_2 mixture, the contents of the side-arm were dumped into the main compartment, the flasks were shaken at a rate of 116 cycles per minute and maintained at 37° C. At the end of the incubation period, 1.5 ml. of the incubation mixture, representing an original content of 1.5 units of insulin, were removed for immediate assay. The slices were dried overnight to constant weight.

The inactivation of insulin during the incubation with liver slices was estimated by the effect of the 1.5 ml. aliquots of the incubation mixture on the blood sugar of rabbits fasted overnight. Blood samples were drawn prior to and at 30 and 60 minutes after the intravenous injection of the incubation mixture. The change in the blood sugar concentration was expressed as the percentage of the average of two fasting sugar levels determined prior to injection. The sum of the percentages of the 30 and 60 minutes blood samples were designated as the "pooled percentage of initial blood sugar" which, in turn, served as a semiquantitative estimate of the insulinase activity of the liver slices. Thus, if no insulin were destroyed during incubation, the "pooled percentage" would be low

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whereas if all the insulin were destroyed, the blood sugar level would remain unchanged and the "pooled percentage" would equal 200. Two sets of an equal amount of slices were prepared from each liver and then assayed as above. The mean of the "pooled percentage" of the two rabbits was used as the estimate of the insulinase activity of each liver.

Two sets of controls were performed; one set consisting of slices incubated in buffer without insulin and the other of buffer with insulin incubated as described but without slices.

Amorphous insulin assaying about 22 units per mg.[2] was utilized. The insulinase-inhibitor was prepared from fresh beef livers by the procedure described in a previous communication (Mirsky, Simkin and Broh-Kahn, 1950). The blood sugar concentration was determined by Nelson's method (Nelson, 1944).

RESULTS

The injection of 1.5 ml. of the phosphate buffer after incubation with liver slices in the absence of insulin produced insignificant changes in the blood sugar of rabbits. Ten sets of slices, ranging from 7.3 to 33.7 mg. dry weight, were incubated for two hours and then assayed as described. The resultant mean "pooled percentage" was 204.3 (S.E. ±0.45) which does not differ significantly from that resulting from the injection of 1.5 ml. of untreated buffer. Accordingly, the incubation of liver slices in phosphate buffer does not release any factor which can produce a change in the blood sugar of rabbits.

TABLE 1. INACTIVATION OF INSULIN BY DIFFERENT PREPARATIONS OF EQUIVALENT AMOUNTS OF LIVER

Homogenates prepared with phosphate buffer, pH 7.4. Supernatant prepared by centrifugation of homogenate at 18,000 for 20 minutes. Slices prepared as described in text. Incubation mixture made up to contain equivalent of 250 mg. liver (wet weight) in total volume of 3 ml. phosphate buffer containing insulin. Rabbits injected with volume of mixture which originally contained 1.5 units.

Preparation	Insulin incubated (units)	Incubation period (minutes)	Insulinase activity ("pooled percentage")
Homogenate	12	30	215
Supernatant	12	30	105
Slices	12	120	87
Slices	3	30	108
Slices	3	120	149
Slices	3	180	200

Assays of 1.5 ml. phosphate buffer containing 1.5 ml. units of insulin after incubation for two hours without liver slices were performed concomitantly with assays of buffer incubated with slices. The mean "pooled percentage of the initial blood sugar" produced by the insulin was 73 (S.E. ±5.7).

In order to evaluate the relative efficiency of homogenates and slices in the inactivation of insulin, preparations of equivalent amounts of liver were studied. As is indicated in Table 1, homogenates and the supernatants after centrifugation at 18,000 g. have a greater insulinase activity than do slices of liver.

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[2] We are indebted to the Eli Lilly Company for generous supplies of insulin.

The relation between the quantity of liver and the inactivation of insulin was determined by incubating insulin with from 1 to 6 slices for 120 minutes. The data obtained in 54 experiments are depicted in Figure 1 from which it is apparent that the greater the quantity of liver, the greater the insulinase activity. In spite of the scatter of the data, the regression coefficient of the insulinase activity ("pooled percentage") on the dry weight of the slices was highly significant ($b = 2.2078$; $P < 0.001$).

The relation between insulinase activity and length of incubation was determined by keeping the quantity of slices constant and varying the period of incubation. The data thus obtained are illustrated in Figure 2 for two different amounts of slices. It is apparent that the rate of insulinase activity, as indicated by the regression of the "pooled percentage" on the time of incubation, is essentially the same for slices weighing 28.0 (S.E. ±0.9) mg. ($b = 0.5406$) as for slices weighing 61.5 (S.E. ±1.3) mg. ($b = 0.5853$), although the amount of insulin destroyed at any particular interval is greater with the larger quantity of liver. Analysis of variance revealed that the differences between the activity at 60 and 120 minutes and between 120 minutes and the other intervals were highly significant ($P < 0.001$).

Since fasting produces a marked diminution in the insulinase activity of liver extracts (Broh-Kahn and Mirsky, 1949), it became pertinent to determine the influence of fasting on the activity of liver slices. Figure 3 illustrates the insulinase activity (mean ± S.E.) obtained with slices from the livers of 40 rats fasted for 72 hours. When slices of less than 30 mg. dry weight were employed no significant difference could be demonstrated between those from livers of fed and fasted rats. With larger quantities of liver slices, however, the activity was significantly higher in those from fasted animals. It is noteworthy that even with the smallest amount of liver, no diminution of activity occurred in slices of liver from fasted rats.

In order to determine the influence of insulinase-inhibitor (Mirsky, Simkin and Broh-Kahn, 1950) on the insulinase activity of slices from the livers of fed rats, 36 experiments were performed with slices incubated for 120 minutes in phosphate buffer containing insulin and slices in buffer containing both insulin and insulinase-inhibitor. The quantity of inhibitor employed in each instance was equivalent to 20 gm. of fresh liver. Figure 4 illustrates the mean ± S.E. activity for slices grouped according to dry weight. Analysis of variance revealed that within each group of slices the presence of inhibitor produced a highly significant diminution in insulinase

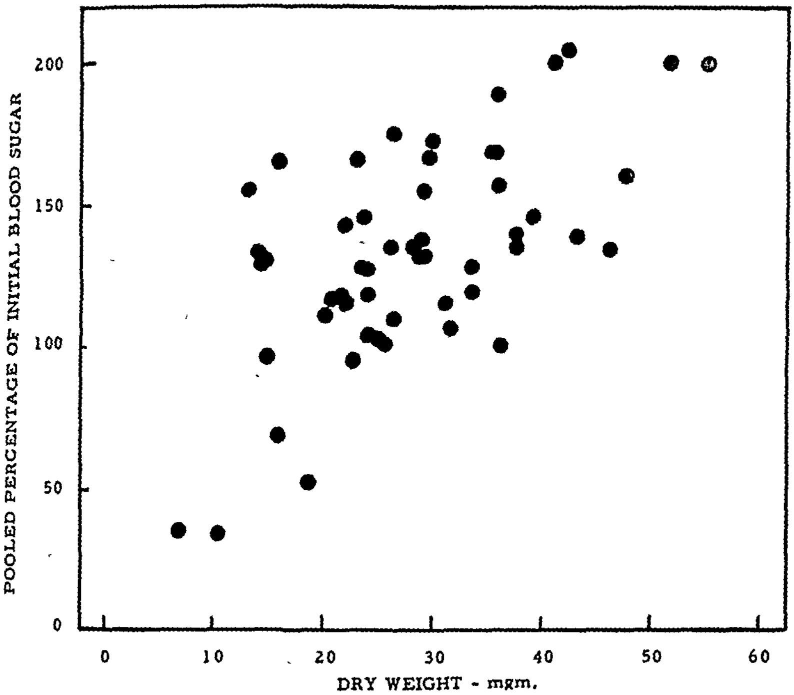

Fig. 1. Relation between the quantity of liver slices and the inactivation of insulin.

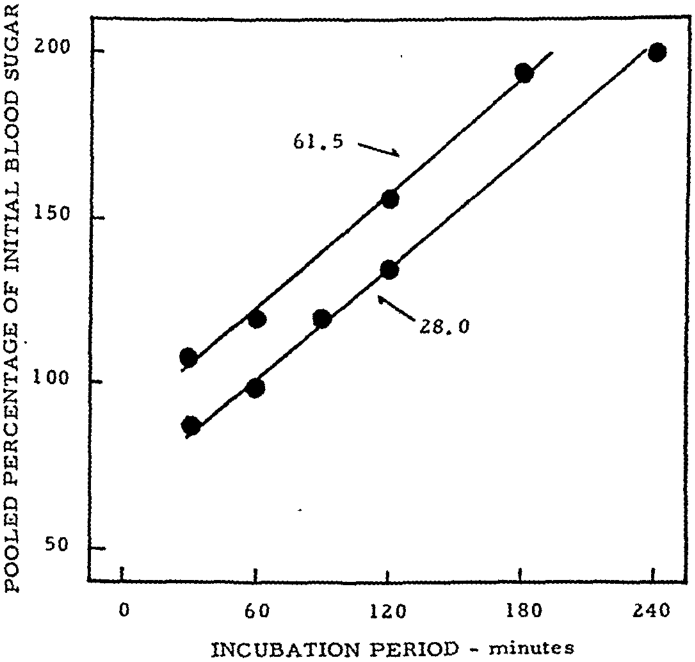

Fig. 2. Relation between duration of incubation and the inactivation of insulin by liver slices.

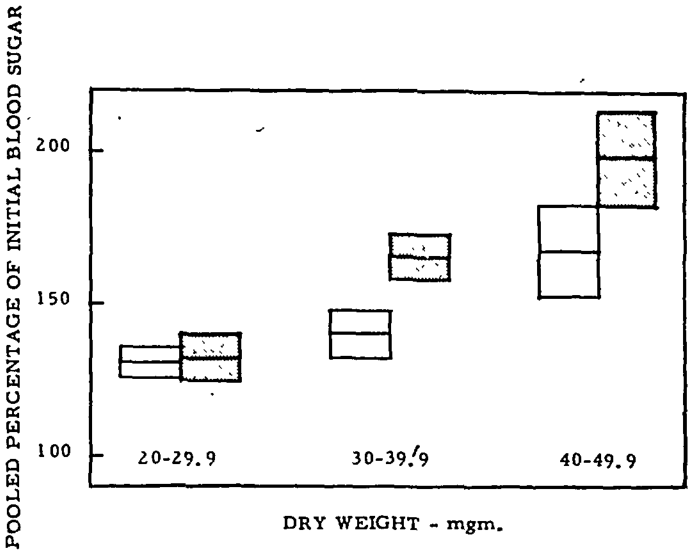

FIG. 3. Effect of fasting on inactivation of insulin by liver slices. The "pooled percentage" (mean ± S.E.) after incubation of insulin with slices from fed rats (open boxes) and with slices from rats fasted 72 hours (cross-hatched boxes).

activity (P <0.001). The same statistically significant difference was established when the dry weights of the liver slices were not grouped and analysis of covariance was employed.

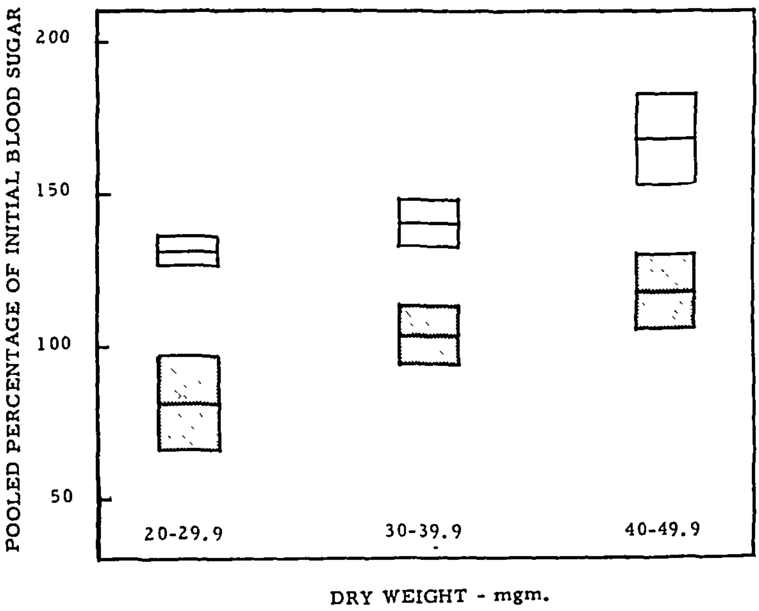

FIG. 4. Effect of "insulinase-inhibitor" on the inactivation of insulin by liver slices. The "pooled percentage" (mean ± S.E.) after incubation of insulin with slices without addition of inhibitor (open boxes) and with slices in presence of inhibitor (cross-hatched boxes).

DISCUSSION

Like homogenates and extracts of liver (Mirsky and Broh-Kahn, 1949), surviving liver slices inactivate insulin *in vitro*. This inactivation is dependent both upon the quantity of liver employed and the length of the incubation period. As with homogenates, the addition of insulinase-inhibitor (Mirsky, Simkin and Broh-Kahn, 1950) to the incubation mixture results in a significant reduction in the insulinase activity of liver slices. With slices, however, the spontaneous rate of insulin inactivation is much slower than with homogenates of equivalent amounts of liver. Consequently, it may be concluded that within the intact cell, only a portion of the potential insulinase is active; the remainder being inhibited by an insulinase-inhibitor.

The insulinase activity of homogenates or extracts from fasted rats is markedly diminished (Broh-Kahn and Mirsky, 1949). Slices of liver from similarly fasted rats, however, exhibit a definite tendency to destroy more insulin than do those from fed rats. Since the inactivation of insulin probably is dependent upon the balance between insulinase and insulinase-inhibitor, the discrepancy between the activity of homogenates and that of slices of livers from fasted rats may be attributed to a greater decrease in the inhibitor than in the insulinase.

It is apparent from the preceding that the assay of homogenates or extracts of liver cannot yield an adequate measure of the rate of insulin inactivation by the intact cell. The slice, however, does provide a measure of the balance between insulinase and insulinase-inhibitor activities, and therefore may be representative of events as they occur *in vivo*. Accordingly, an increase in the rate of insulin inactivation should occur in the intact fasted rat. Since insulin production by the pancreas is decreased during fasting (Best, Haist and Ridout, 1939), the increased rate of insulin inactivation should result in a relative insulin insufficiency. Further, with an increase in insulinase activity, exogenous insulin should be less effective since only a portion of the quantity administered will escape inactivation.

The aforementioned postulates are in accord with established data concerning the phenomenon of "starvation diabetes." Thus, the fasted animal not only responds to the ingestion of carbohydrate with a typical "diabetic tolerance" curve, but also shows a decreased hypoglycemic response to the injection of insulin (Chambers, 1938).

The relevance of insulinase and insulinase-inhibitor to the etiology of diabetes mellitus in man is difficult to evaluate at the present time. The presence of these factors in extracts of liver and the demonstration that they are effective in the intact cell lends support to the hypothesis that an insufficiency of insulin may result from an increased destruction of insulin either in consequence of an increase in insulinase activity or of a decrease in the availability of the insulinase-inhibitor (Mirsky, 1951).

SUMMARY

The insulin-inactivating activity (insulinase) of slices of liver is much less than that of homogenates or extracts of equivalent amounts of liver.

Insulinase activity is related directly to the quantity of liver slices employed and to the length of incubation.

In the presence of an inhibitor extracted from liver (insulinase-inhibitor), the insulinase activity of liver slices is markedly reduced.

Whereas fasting results in a diminution in the insulinase activity of homogenates and extracts of liver, slices of liver from fasted rats tend to have an increased activity. It is proposed that the insulin-inactivating capacity of liver slices is dependent upon the balance of insulinase and insulinase-inhibitor activities, and that with fasting there is a greater reduction in the inhibitor than in the insulinase.

The phenomenon of "starvation diabetes" may be atrributed in part at least to a relative increase in insulinase activity.

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THE THYROID STIMULATING HORMONE OF THE ANTERIOR PITUITARY AS DISTINCT FROM THE EXOPHTHALMOS PRODUCING SUBSTANCE[1]

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FOR almost two decades it has been known that anterior pituitary extracts when injected into animals produce hyperplasia of the thyroid and exophthalmos. Probably owing to the frequent coexistence of these two features in patients with Graves' disease, it has been generally assumed that the hyperplasia of the thyroid and the exophthalmos in the experimental animals were caused by the same pituitary factor, the thyroid stimulating hormone (TSH).

From observations (Dobyns, 1946a, 1946b) on the exophthalmos producing activity of a variety of TSH preparations, it has become apparent that more striking exophthalmos was produced by some extracts than others although hyperplasia of the thyroid was produced by all. It has been reported by Jefferies (1949) that following the iodination of pituitary extract the thyroid stimulating material was inactivated but some exophthalmos producing activity remained. On the other hand, Dobyns and Rawson (1951) have found that an extract rich in TSH when exposed to thyroid tissue slices *in vitro* lost the capacity to produce both responses.

Albert (1945) has shown that the Fundulus, the common Atlantic "minnow," (*Fundulus heteroclitus, Linn.*) will develop exophthalmos following the administration of small amounts of anterior pituitary extract.

Albert (1945) tested a variety of prepared endocrine substances as well as six anterior pituitary hormones (in various degrees of purification) which he had prepared from pituitaries of sheep. He observed that the exophthalmos producing activity paralleled only the thyrotropic activity and that preparations of follicle stimulating hormone and luteinizing hormone which produced exophthalmos also caused hyperplasia and hypertrophy of the thyroid.

The purpose of this study has been to determine the exophthalmos producing quality of a large number of anterior pituitary fractions which

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had been prepared in a variety of ways and for a variety of purposes and for which some assay data concerning some anterior lobe hormones were available. It became apparent from the study of some preparations that the exophthalmos producing and the thyroid stimulating activities did not necessarily parallel one another. These observations have led to the separation of thyroid stimulating hormone (TSH) from the exophthalmos producing substance (EPS).

METHODS

Groups of 8 to 12 Fundulus were placed in stainless steel tanks. Three to six groups of fish were used in each experiment. One group of fish received normal saline; one group of fish received a standard pituitary extract for which a known exophthalmos producing response had been determined; and the remaining groups received various unknown preparations.

The material to be tested was weighed and then dissolved in normal saline. A single dose of 0.25 mg., or a fraction thereof, in 0.25 ml. of saline was injected intraperitoneally by introducing a #26 needle through the vent and the wall of the bowel. Many fractions and subfractions of pituitary extracts rich in various trophic substances as well as the by-products of purification procedures were tested.

Colored thread was placed through and tied in the lower lip of each fish so that individual identification was possible. The intercorneal distance on each of the fish was measured to 0.1 mm. by means of an improved caliper method modified after that described by Dobyns (1945). Such measurements were made on each fish before and at intervals following the administration of the material tested. The degree of exophthalmos was calculated as the percentage increase in the intercorneal distance in each fish. The average percentage change among the fish of each group was thus obtained.[2]

The amount of TSH in the various preparations was determined in day-old, male, white leghorn chicks by one or several modified methods: 1) the increase in thyroid weight (Bergman and Turner, 1939); 2) the depletion of iodine from the thyroid (Piotrowski *et al.*, 1953); 3) the increase in mean cell height of the thyroid (Rawson and Salter, 1940).

RESULTS

As a result of screening a large number of pituitary fractions, it was observed that a few fractions which contained appreciable amounts of TSH produced relatively little exophthalmos; conversely, a few preparations which contained relatively little TSH produced obvious exophthalmos. Some of the results of these assays are shown in Table 1. See Figure 1.

With the above assay data available, an analysis of the various chemical procedures used in the preparation of these pituitary fractions pointed the way to a method for specifically separating the TSH and the EPS in appropriate pituitary extracts. Since thyrotropin preparation No. 11

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[2] This method of assay differs from that of Albert (1945) in two ways. Albert classified the exophthalmic response as present or not present in each fish when grossly compared to controls. The value for each group of fish was recorded as the percentage of positive responses.

Table 1. The assay of exophthalmos producing substance (EPS) in a variety of pituitary fractions[1]

Unknown preparation	Constituents[2] known to be present	Av. % increase intercorneal distance		
		Unknown	KD44A (standard)	Saline control
1	TSH 83%	14.3±2.7	16.4±1.5	0.5±1.0
		15.1±2.3	15.5±2.3	0.5±1.7
2	TSH 38%	5.3±1.8	16.4±1.5	0.5±1.0
3	TSH 35%	2.5±1.0	16.4±1.5	0.5± .0
4′	TSH 28%	2.4±1.1	19.0±3.3	2.8±2.4
5	TSH 45%	8.3±0.9	19.0±3.3	2.8±2.4
6	TSH 55%	15.0±3.0	16.1±2.8	2.8±1.8
7	TSH 10%; LH high; FSH low	19.9±2.7	16.1±2.8	2.8±1.8
		19.2±0.59	15.6±2.0	−0.9±0.6
8	TSH low; LH low; Prolactin high	3.0±0.8	14.3±2.3	2.6±0.7
		4.8±1.5	11.8±1.7	2.5±0.2
9	TSH 150–200%; LH low; Prolactin low	6.2±1.2	11.8±1.7	2.5±0.2
10	TSH 30%; LH low; FSH high	3.8±1.2	14.1±1.9	−0.7±0.8
11	TSH 1000%; LH low	2.2±2.6	13.1±2.6	1.2±1.0
12	TSH 10%; LH high	10.5±0.6	5.3±2.7	0.5±1.1
13	ACTH moderate	0.1±1.6	12.5±1.8	—
14	TSH 100%; LH low; Growth low; ACTH low	19.4±2.1	17.8±2.1	0.5±1.4
15	TSH 300%; LH low; Growth low; FSH low	18.4±2.4	16.5±2.7	−2.1±1.5
16	Growth high	0.2±2.0	16.4±1.5	0.5±1.0
17	TSH 100%; LH very high	17.7±2.5	16.8±1.9	0.6±0.47
18	FSH high; TSH <10%; ACTH 0.0096 USP μ/mg.; Growth nil	0.5±0.56	10.0±1.36	1.0±0.85
19	TSH very low; LH high; FSH high	7.4±3.17	14.8±4.44	0.6±1.81

It will be noted that in Experiments #7, #11, #12, and #19, the known potency of TSH did not coincide with the exophthalmos producing activity demonstrated.

[1] 0.25 mg. of powder used per fish.

[2] TSH = Thyroid Stimulating Hormone potency, recorded as per cent of Armour standard 2R3 (see #14) which is regarded as 100%; LH = Luteinizing Hormone; FSH = Follicle Stimulating Hormone; ACTH = Adrenocorticotropic Hormone; and Growth = Growth Hormone.

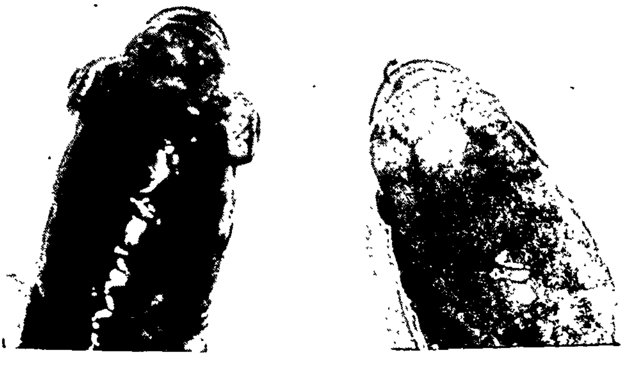

Fig. 1. An example of the exophthalmic response produced in Fundulus by the administration of an anterior pituitary extract rich in the exophthalmos producing substance (EPS). The intercorneal distance of the fish on the left increased 13.2% in 6 hours. The fish on the right served as a control.

TABLE 2. ASSAYS OF EXOPHTHALMOS PRODUCING SUBSTANCE (EPS) IN THE RESIDUE (R) AND SOLUBLE (S) FRACTIONS PREPARED FROM PITUITARY EXTRACTS KNOWN TO HAVE A CAPACITY TO STIMULATE THE THYROID AND TO PRODUCE EXOPHTHALMOS

Preparation	Average percentage increase in intercorneal distance[1]			
	Unknown		KD44A (standard) 0.25 mg.	*Saline* (control)
	0.25 mg.	0.125 mg.		
15[2]	14.5 ±2.1 (18.4 ±2.4)	10.5 ±1.8 (12.4 ±1.7)	11.8 ±1.7	0.59 ±1.6
183S	0.3 ±0.55	−0.7 ±1.0	11.7 ±1.7	0.59 ±1.6
183R	13.0 ±1.52	7.2 ±1.07	11.7 ±1.7	0.59 ±1.6
IRW[2]				
$224S_1$	−1.4 ±0.71		9.7 ±1.82	0.14 ±0.82
$224S_2$	−0.9 ±0.79		9.7 ±1.82	0.14 ±0.82
$224R_2$	7.6 ±1.7		9.7 ±1.82	0.14 ±0.82

Compare the results of the EPS assays on the residue (R) and soluble (S) fractions appearing in this table with the results of the TSH assays on the same fractions appearing in Table 3. EPS is contained in the R fractions.

[1] All percentages are expressed as a positive increase unless specified by a minus sign. Maximal increase in intercorneal distance usually occurred about 6 hours following injection. Therefore, the data acquired in 6 hours have been recorded. In instances where further increase was observed at 17 hours, the values have been recorded in parentheses.

[2] Starting material (same preparation as No. 15, Table 1).

(Table 1) contained little if any exophthalmos producing activity, yet the material from which it was derived was rich in it; and since it represented the soluble fraction from trichloracetic acid precipitation, this method was applied to a new starting material, pituitary extract No. 15, which had been found to produce both exophthalmos and thyroid hyperplasia.

Beef thyrotropin (No. 15 in Tables 1 and 2) was dissolved in distilled water to make a 1% solution. Trichloracetic acid[3] was added to make a concentration of approximately 8%. After thorough stirring, the mixture was allowed to stand at 3–5°C. for 1 hour. Following centrifugation the precipitate was separated from the supernatant, taken up in water (one-half of the original volume), brought to neutrality by the addition of sodium carbonate, dialyzed until free of salt, and lyophilized. The supernatant solution, that had resulted from the above trichloracetic acid precipitation, was also neutralized, dialyzed, and lyophilized. The activities of EPS and TSH in the residue (183R) and in the supernatant (183S) were assayed. The EPS was entirely confined to the residue fraction (183R) and the TSH was predominantly in the soluble fraction (183S). The results of the assay for EPS in each fraction are shown in Table 2. The results of several methods of assay for TSH in these fractions are shown in Table 3.

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[3] Trichloracetic acid precipitation has been used by some authors (Loeser, 1932; Ciereszko, 1945; Albert, 1949) as a step in the purification of thyroid-stimulating activity of pituitary extracts; however, in their purification procedure the TSH was recovered in the supernatant fluid and the precipitate served to remove materials other than TSH.

TABLE 3. ASSAYS FOR TSH IN FRACTIONS LISTED IN TABLE 2

	Total dose/chick (gamma)	Av. chick thy. wt.[1]	Control thy. wt.[1]	Mean cell ht. (microns)[2]	Iodine depletion[3]
				Control (2.89 ± .040)	
15[4]	50	4.19 ± .44	3.53 ± .22		
	100	6.35 ± .49	3.53 ± .22		1.2–1.3 u/mg.
	200	6.85 ± .48	3.53 ± .22		
	400	7.13 ± .45	3.98 ± .15	5.38 ± .038	
183S	50	5.35 ± .30	3.53 ± .22		
	100	5.72 ± .40	3.53 ± .22		3.5 u/mg.
	200	5.11 ± .44	3.53 ± .22		
	400	6.88 ± .53	3.98 ± .15	5.02 ± .02	
183R	50	4.35 ± .29	3.53 ± .22		
	100	4.82 ± .41	3.53 ± .22		0.6 ±0.8 u/mg.
	200	5.81 ± .28	3.53 ± .22		
	400	5.50 ± .36	3.98 ± .15	3.79 ± .036	
IRW[5]				Control (2.73 ± .034)	
$224S_1$	100	6.49 ± .28	3.97 ± .18	5.25 ± .034	2.0 μ/mg.
$224S_2$	100	8.26 ± .45	3.97 ± .18	5.84 ± .063	3–4 μ/mg.
$224R_2$	100	4.78 ± .16	3.97 ± .18	3.40 ± .033	0.25 μ/mg.

Compare the results of the TSH assays on the residue (R) and soluble (S) fractions appearing in this table with the results of the EPS assays on the same fractions appearing in Table 2. Most of the TSH is in the S fraction. It was shown in Table 2 that EPS was confined to the R fraction.

[1] Based on 9 to 11 chicks per group.

[2] Based on 7 to 10 thyroids in each group. A representative cell in each of 50 acini was measured in each gland.

[3] USP units.

[4] Starting material (same preparation as No. 15, Table 1).

[5] Starting material.

Although TSH was thus obtained free from EPS, the EPS fraction contained some TSH. In order to confirm the above observations and to improve the purification of the EPS, another new starting material (pituitary extract, No. IRW) was treated in the same manner as described above except that the precipitate produced by trichloracetic acid (residue fraction No. $224R_1$) was suspended in enough water to effect solution and reprecipitated with trichloracetic acetic acid as above. The double precipitation procedure thus yielded two soluble fractions (1st, No. $224S_1$; 2nd, No. $224S_2$) and one residue fraction ($224R_2$). In Tables 2 and 3, it may be seen that the two soluble fractions ($224S_1$ and $224S_2$) contained TSH free of EPS, and that the reprecipitated residue fraction ($224R_2$) contained EPS which was relatively free from TSH. It is also evident that considerable TSH was removed from the first residue fraction by the re-precipitation procedure.

DISCUSSION

The experimental evidence which has been presented indicates that when anterior pituitary extracts are administered to animals, TSH *per se* is not responsible for the development of exophthalmos. It appears that there is another substance which produces the exophthalmos. We have proposed to call this exophthalmos producing substance (EPS).

These observations give support to the suspicion registered by one of the authors (Dobyns, 1946a, 1946b) and Jefferies (1949) that TSH might not be the substance which produces exophthalmos. Since known as well as unknown factors still remain in most anterior pituitary preparations, it is possible that a substance which inhibits exophthalmos may be removed from some preparations while being concentrated in others. This question is raised because thus far the EPS preparations still appear to have some TSH impurities in them. The possibility of an inhibitory substance being responsible for these findings seems very unlikely since it is possible to eliminate EPS from TSH and to eliminate TSH almost entirely from EPS (see Tables 2, 3; 224 series).

The theory that TSH *per se* is responsible for clinical exophthalmos has not been consistently supported by the results of assays in clinical subjects (Starr and Rawson, 1937; Purves and Griesbach, 1949). The failure to show a high titre of TSH in all patients suffering from progressive exophthalmos has perhaps been more frequently attributed to the inadequacy of the assay methods than to the possibility that TSH might not be responsible for the problem concerning the eyes. As a result of the findings described here, an assay method for the exophthalmos producing substance in selected clinical subjects is now being explored.

SUMMARY

It has been found in a variety of pituitary extracts studied that the thyroid stimulating hormone (TSH) and the exophthalmos producing substance (EPS) in anterior pituitary extracts do not always parallel one another. An analysis of the various chemical procedures by which these preparations were obtained lead to the separation of TSH from EPS. These two substances were separated by their differential solubility in trichloracetic acid. EPS was assayed in Fundulus and TSH was assayed in day-old chicks. The results indicate that EPS and TSH are not the same substance.

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RESPONSE OF INBRED LINES OF CHICKENS TO THIOURACIL[1]

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IT HAS long been known that there are wide species, sex and age differences in thyroid activity. It is also known that environmental factors such as season and temperature, iodine intake, pituitary activity, administration of goitrogens and the alteration of endocrine activity by other means may have a profound effect on thyroid function. In recent years evidence has been accumulating with respect to the influence of genetic makeup or constitution on thyroid function within species.

During the past half century poultry breeders have greatly improved the type, productivity and efficiency of the various breeds of chickens such as the White Leghorn, Barred Plymouth Rock, Rhode Island Red, New Hampshire and others. In addition, they have developed strains or lines of these breeds which are especially noted for such characteristics as egg number, egg size, rate of growth, rate of feathering, and so on. With such changes in phenotype and genotype, it is only logical to attempt to correlate these differences with specific physiological characters.

Several investigators have reported breed and strain differences in thyroid activity of chickens. Schultze and Turner (1945) found that thyroxine secretion rate on a body weight basis was greater in White Leghorn males than in White Plymouth Rock males. Mixner and Upp (1947) compared single cross hybrid parents with their 4-way cross hybrid progeny, and found increased thyroxine secretion rate in the hybrid offspring. They suggested that increased thyroxine secretion rate might be a factor in heterosis or hybrid vigor. Glazener, Shaffner and Jull (1949) found that thyroxine secretion rate, measured only in female chicks, was higher in rapid-growing strains. Thyroprotein depressed growth in slow-growing New Hampshires and rapid-growing Barred Rocks. It was concluded that "each strain had a physiological optimum level of thyroid activity for growth, feed utilization and feathering." El-Ibiary and Shaffner (1950) selected chicks for large and small thyroid size; selection was on a family

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basis from birds fed a normal ration. The chickens from lines selected for large and small thyroid size were then fed thiouracil and it was possible to develop lines which differed in responsiveness to thiouracil. Boone *et al.* (1950) measured thyroxine secretion rate in fast- and slow-feathering Rhode Island Reds. Thyroprotein administration increased rate of feathering in the slow-feathering strains. The daily d,l-thyroxine secretion rate was slightly higher in the slow-feathering birds, although correction for body weight showed thyroxine secretion to be somewhat higher per unit of body weight in the fast-feathering chickens. These authors concluded that the evidence available does not indicate that slow-feathering is due to an inherently low thyroid secretion rate.

The experiments reported in this study were carried out to compare various inbred lines of chickens in response to thiouracil.

PROCEDURE

Five distinct lines of Leghorn chickens, obtained from a commercial source, were used.

Two separate pens of parent stock of line 1 were maintained. The chicks from these pens were designated as lines 1A and 1B and were supposedly of identical genetic make-up. The amount of inbreeding of each was, therefore, the same—37½%. Line 2 had been inbred for seven generations and had an inbreeding coefficient of 78%. Line 3 also had an inbreeding coefficient of 78%, and line 4 an inbreeding coefficient of 59%. Line 5 was non-inbred. The 5 lines were not of common origin.

Trials I and II were replications in successive weeks. In trial III a comparison was made of the variability within the highly inbred line 2 and line 1A which had an inbreeding coefficient approximately half as great (37½%). In trial IV comparisons were made of crosses of 3 of the lines studied in the first 3 trials. It was anticipated that the 3 crosses would be similar; two crosses were reciprocal matings of lines 1 and 2. There was also a cross of line 1 with line 3. Line 3 was similar to line 2 in 13-day gain, thyroid weight and relative thyroid weight, as may be seen in Table 1. Thus it was expected that the cross of lines 1 and 3 might be similar to the crosses of lines 1 and 2.

The chicks were received on the second day after hatching. They had been sexed and were males, with the exception of a negligible number of females. Half the chicks of each group were fed a standard chick starting ration and the remainder received the same ration to which thiouracil had been added at a level of 0.2%. The initial weight of each chick was taken. At the end of 13 days the chicks were again weighed and were then killed and the thyroid glands removed and weighed.

The data analyzed were: 13-day gain in grams, thyroid weight in milligrams, and relative thyroid weight (thyroid weight as a percentage of body weight).

The gains and the thyroid weights were analyzed as grams and milligrams, respectively, and also as logarithms of those values. The object of making both analyses was to learn whether certain effects tended to be constant in absolute units or percentage-wise. No clear-cut conclusion is possible regarding the form in which the data have homogeneous variance, since the evidence regarding homogeneity is conflicting in the various trials with the data in either form. However, the same conclusions were reached regarding the significance of line and diet effects regardless of the form in which the data were analyzed.

TABLE 1. EFFECT OF THIOURACIL ON THYROID WEIGHT AND RATE OF GAIN IN TRIALS I AND II[1]

Line	Inbreeding coefficient	Thiouracil treatment	Thyroid weight, mg.	Thyroid wt. as % of body wt.	13-day gain, gm.
1 A	37.5%	None	9.8	0.0089	75.2
		0.2%	26.1	.0295	52.5
1 B	37.5%	None	10.3	.0096	71.6
		0.2%	24.0	.0290	49.8
2	78.0%	None	8.2	.0099	50.0
		0.2%	13.1	.0192	35.8
3	78.0%	None	7.5	.0093	51.1
		0.2%	14.9	.0223	34.9
4	59.0%	None	9.8	.0095	67.0
		0.2%	18.5	.0217	49.1
5	0	None	8.8	.0074	84.8
		0.2%	25.5	.0268	58.3

[1] The number of chicks of a line on a diet varied from 21 to 25.

TABLE 2. A COMPARISON OF TWO INBRED LINES IN RESPONSE TO THIOURACIL, TRIAL III

Line	No. of chicks	Inbreeding coefficient	Thiouracil treatment	Thyroid weight, mg.	Thyroid wt. as % of body wt.	13-day, gain gm.
1 A	22	37.5%	None	13.7	.0124	78.9
	43		0.2%	33.6	.0359	58.9
2	21	78.0%	None	9.1	.0102	61.6
	38		0.2%	18.7	.0272	38.5

RESULTS AND DISCUSSION

The data for all 4 trials are summarized in Tables 1, 2, 3 and 4.

Thyroid weight

As expected, thiouracil produced a highly significant ($P < 0.01$) increase in thyroid weight in all 4 trials. In each of the 3 trials of inbred lines, the lines differed highly significantly in thyroid weight. Also as expected, the 3 crosses in trial IV had similar thyroid weights: These weights were very similar for the reciprocal crosses. The weight differences were also small between the reciprocal crosses and the third cross. All of these differences were so small that they were far from significant.

In each of the 4 trials, the increase in thyroid weight due to thiouracil varied from inbred line to inbred line or from cross to cross. (In other words, there was diet and line (or cross) interaction.) These variations (interactions) were highly significant whether the analysis was in milligrams or logs. In the 4 trials, the weight of the thyroid with thiouracil was 194% to 241% of the weight without.

TABLE 3. EFFECT OF THIOURACIL ON LINE CROSSES, TRIAL IV[1]

Line cross	Thiouracil treatment	Thyroid weight, mg.	Thyroid wt. as % of body wt.	13-day gain gm.
1B×2	None	11.0	.0106	73.0
	0.2%	25.3	.0288	56.0
2×1A	None	11.0	.0102	74.1
	0.2%	24.0	.0265	54.7
1A×3	None	9.6	.0097	72.5
	0.2%	29.7	.0342	57.5

[1] The number of chicks of a cross on a diet was 12 or 13.

TABLE 4. VARIATION OF CHICKS OF TWO INBRED LINES WITH DIFFERENT INBREEDING COEFFICIENTS, TRIAL III

Line	Inbreeding coefficient	Thiouracil treatment	Standard deviation	Coefficient of variation
		13-day gain		
1 A	37.5%	None	8.9 gm.	11%
2	78%	None	7.9 gm.	13%
1 A	37.5%	0.2%	5.9 gm.	10%
2	78%	0.2%	5.6 gm.	15%
		Thyroid weight		
1 A	37.5%	None	4.1 mg.	29%
2	78%	None	3.8 mg.	41%
1 A	37.5%	0.2%	8.8 mg.*	26%
2	78%	0.2%	4.8 mg.*	26%

* These 2 standard deviations differ significantly ($P < 0.2\%$). The members within the other 3 pairs of standard deviations are not significantly different.

For thyroid weight, the 5 lines performed similarly with respect to each other in the various trials. There was moderate—but nonsignificant—evidence of diet and trial interaction for thyroid weight; the increase due to thiouracil was least in trial II and most in III.

Relative thyroid weight

No tests of significance were made of effects on relative thyroid weight, but some conclusions appear justified. Relative thyroid weight was increased greatly by thiouracil in all 4 trials and with all lines and crosses. However, the effect of thiouracil varied considerably among lines and among crosses. In trials I and II, the relative thyroid weight was increased least in line 2, more in lines 3 and 4, and most in line 5 and in the 2 groups of line 1. In trial III also, the effect was considerably less in line 2 than in 1. In trial IV the effect was greater in the cross of lines 1 and 3 than in the reciprocal crosses of lines 1 and 2.

13-day gain

In the selection or development of strains or lines of various domestic animals, growth rate is of great economic importance. As expected, the induction of hypothyroidism with thiouracil had a highly significant depressing effect on rate of gain in all lines and crosses in all 4 trials. The lines (in trials I, II and III) differed highly significantly in gain. See Tables 1 and 2. Line 5 gained most rapidly; line 1 was second in gain, and lines 2 and 3 gained least. On the control diet, line 5 gained 70 % more than line 2, and the average gain of the 2 groups of line 1 was 56 % greater than the gain of line 2. As anticipated, the crosses in trial IV differed little in gain.

When gains in grams were analyzed, it was found that the depression from thiouracil was significantly different for the various lines, i.e., there was significant diet and line interaction. However, the evidence of interaction was negligible for logs. This means that the depression was a nearly constant percentage of the gain without thiouracil. In each trial the depression was close to 30 %. From trial to trial, also, the depression in gain due to thiouracil was 30 %, but the depression in grams was different for trials in which the gain on the control diet differed.

Relation of gain and thyroid weight

Coefficients of the relation of gain and thyroid weight were not calculated for chicks of the same inbred line because dot diagrams indicated no appreciable relation. However, correlation coefficients were computed using inbred line averages. One of the variables correlated was the average gain in grams on both diets; the other was the average thyroid weight in milligrams on the thiouracil diet. The correlation coefficient was 0.92.

The comparative variation of line 1 (inbreeding coefficient 37.5%) and line 2 (inbreeding coefficient 78%) was studied in trial III. Only for these 2 lines and only in trial III was it possible to calculate the variation of chicks with the same sire and dam. Table 4 shows standard deviations and coefficients of variation. In all 4 cases (i.e., 13-day gain and thyroid weight on each of the 2 diets) line 1A varied more than line 2, although the difference in the standard deviations was statistically significant (with $P < 0.2\%$) only for thyroid weight on the thiouracil diet.

There were too few sires and dams to make worthwhile estimating the components of chick variance due to sires and dams.

Comparison of lines and their crosses

It seems reasonable to compare lines 1 and 2 in trial III with their reciprocal crosses in trial IV, since these trials were largely concurrent. (Ten of 13 days were common to the 2 trials.) On each diet, the gain of the reciprocal crosses was between the gains of the 2 parental lines, but was closer to the gain of the faster-gaining line, 1. This suggests some hybrid vigor. The respective average gains in grams for line 1, the reciprocal crosses, and line 2 were: 79, 74, and 62 on the control diet, and 59, 55, and 39 with thiouracil.

Thyroid weights of the reciprocal crosses were more nearly midway between the weights for the parents. The average thyroid weights in milligrams for line 1, the crosses, and line 2 were: 14, 11, and 9 on the control diet, and 34, 25, and 19 with thiouracil.

Relative thyroid weights of the crosses were similar to those of the parent with the lower relative weight. The values in percentage in the same order as previously given were: 0.0124, 0.0104, and 0.0102 with the control diet, and 0.0359, 0.0276, and 0.0272 with thiouracil.

These studies provide additional evidence of endocrine differences between lines or strains of the same species. They are similar in general nature to the conclusions of Boettiger (1941), Koger *et al.* (1942), Hurst and Turner (1948) and Stob and Andrews (1952) that strains of mice differ in thyroid physiology. They likewise support the conclusions of Mixner and Upp (1947), Glazener *et al.* (1949), El-Ibiary and Shaffner (1950) and Boone *et al.* (1950) that strains or lines of chickens vary widely in thyroid function.

In any breeding program it is desirable to determine the characteristics of the progeny at the earliest possible time and during the shortest practical period of observation. If strain differences in thyroid function are important, and there is every reason to believe thay are, it seems obvious that they can be detected in the young chick (14 days of age) and without elaborate procedures. Highly significant differences in thyroid weight and in 13-day gain were readily detected. An extremely high correlation of rate of gain and thyroid weight on the thiouracil diet was found ($r=0.92$).

Since one of the basic principles of selection and breeding is the reduction in variability, it was of interest to measure the effects of inbreeding upon such specific factors as thyroid size and response to thiouracil. In general there was less variation in a line with an inbreeding coefficient of 78% as compared with one of 37½%.

From the genetic standpoint, the comparisons of lines and their crosses is of special interest. Gross thyroid weight of the reciprocal crosses was about midway between the weights of the parents. When thyroid weights was expressed as a percentage of body weight, the relative thyroid weights of the crosses were similar to those of the parent with the lower relative gland weight.

SUMMARY

Five lines of chickens, varying in inbreeding coefficients from 0 to 78%, were fed thiouracil at a level of 0.2% of the diet from the second to the 14th days post-hatching. There were considerable differences between lines in gross and relative thyroid weight. Growth rate, expressed as 13-day gain, varied highly significantly between lines. Thiouracil had a highly significant depressing effect on rate of gain. Thiouracil depressed the growth rate about 30%. Thus the rate was depressed more *in grams* in a fast-growing line than in one which grew slowly. Using inbred line averages, the correlation coefficient of 0.92 was obtained when 1 variable was the average gain in grams on both diets and the other variable was the average thyroid weight in milligrams on the thiouracil diet.

Thyroid weight and 13-day gain of chicks with the same sire and dam varied more in line 1 (37.5% inbred) than in line 2 (78% inbred).

Line 1 and 2 and their reciprocal crosses were compared. The gains of the reciprocal crosses were between the gains of the two parental lines but closer to the gains of the faster-gaining line, 1. Gross thyroid weights of the crosses were nearly midway between the gland weights of the parents. Relative thyroid weights of the crosses were similar to those of the parent with the lower relative thyroid weight.

These studies provide additional evidence of endocrine differences between lines or strains of the same species.

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NOTES AND COMMENTS

SURVIVAL OF MATING BEHAVIOR IN MALE RATS AFTER THYROID-PARATHYROIDECTOMY[1]

Young, Rayner, Peterson and Brown (1952) have recently reported that thyroidectomy does not alter the copulatory behavior of male guinea pigs. Their study included objective and quantitative measures of coital activity before and after operation. No comparable evidence is available with respect to the male rat and such casual observations as have been reported are contradictory, some writers stating that loss of the thyroid eliminates or greatly reduces sexual responses (Rickey, 1925) and others asserting that the behavior is unchanged (Hammett, 1922). The present investigation was conducted to measure pre- and postoperative behavior in standardized fashion.

SUBJECTS AND METHODS

Thirteen adult male albino rats 90 to 150 days old at the start of the experiment were given a series of preoperative mating tests with receptive females. The method of testing for sexual vigor has been described in earlier publications and has been proven to be highly reliable (Beach, 1944). The most important responses recorded are incomplete copulations, intromissions, and ejaculations. Each animal was tested until he had copulated (but not necessarily ejaculated) in a minimum of 3 tests. The thyroid and parathyroid glands were removed, 0.5% calcium gluconate was added to the stock diet, and 8 postoperative mating tests were conducted in the course of the next 30 days. For 30 days after the final test 0.2% thiouracil was added to the diet in the expectation that if the operation had been incomplete any residual glandular tissue would hypertrophy and be more easily discovered in the postmortem examination. When the neck region was examined under low magnification small tabs of tissue which might have been thyroid were discovered in only 3 rats, and in each case these fragments were sectioned and stained and proved to be nonglandular.

RESULTS AND CONCLUSIONS

Comparison of mating performance in pre- and postoperative tests revealed no significant differences. Three males which had not ejaculated before operation did so afterwards and one case failed to ejaculate postoperatively although this response had occurred previously. The average frequency of intromissions was 7.0 per test before and 7.4 after operation, a difference which was not statistically significant. The frequency of incomplete copulations did not change significantly.

These findings are in harmony with the thesis that secretions of the thyroid and parathyroid glands are not essential to normal mating behavior in the male rat.

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